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BACTERIAL AND MYCOTIC  
INFECTIONS OF MAN





# BACTERIAL AND MYCOTIC INFECTIONS OF MAN



*Edited by*  
RENÉ J. DUBOS, Ph.D.  
*The Rockefeller Institute for  
Medical Research*



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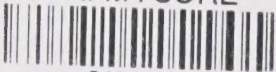
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# Preface

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This volume was designed to convey to the medical student—and we hope also to the practitioner of medicine—some knowledge of the bacteria, actinomycetes and molds pathogenic for man, as well as of the phenomena which characterize the infectious process. Infections caused by viruses and rickettsiae are treated in a companion volume edited by Dr. T. M. Rivers.

Medical microbiology is the study of host-parasite relationships and not that of microorganisms alone, considered as independent living agents. It is concerned with those aspects of the structure and the properties of parasites which play a part in their pathogenic behavior, and with the multiple manifestations of the response of the invaded host to their constituents and products. The general chapters of this treatise are therefore devoted to the facts and the problems concerning parasite and host which have a bearing—often immediate, but at times only potential and remote—on infectious disease.

A few words may be necessary to justify the order in which the different pathogenic agents are described in subsequent chapters. This order was adopted to illustrate, by the extensive treatment of a few selected examples, the multiple facets of the problem of infection. Thus, the diphtheria bacillus is discussed first to introduce the concept of toxemia and of antitoxic immunity.

As a counterpart, pneumococcus infections are then selected to emphasize the problems of antibacterial immunity. Streptococci, on the other hand, lend themselves to the demonstration that a given microbial agent can exhibit multiple pathogenic potentialities, and that tissues can respond in many different ways to its presence. Tuberculosis illustrates particularly well the acute (exudative) and chronic (proliferative) pathologic processes accompanying infection, and the altered reactivity of the body (allergy) which results from previous exposure to the bacillus. All these aspects of the infectious process appear in more-or-less modified form in the other microbial diseases and give to each of them its peculiar character.

This treatise is the result of the co-operative effort of many experts and naturally reflects their individual outlooks. I wish to thank them all, in particular for their willingness to aim at some measure of uniformity in our common undertaking. The National Foundation for Infantile Paralysis has given generous financial support to the preparation of the book and shares with us the hope that it may contribute something to the understanding of the general problems of infection.

RENÉ J. DUBOS

The Rockefeller Institute  
for Medical Research  
New York





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# 1

## A Synopsis of the History of Medical Bacteriology\*

The history of bacteriology is brief but crowded with infinitely varied significance. The discoveries and applications of less than a hundred years did more than modify the conceptions and theories built by the scientific endeavours of preceding centuries, they formed a freshly new branch of biologic science, *bacteriology*, and such great progress was made that there emerged from it the two further highly specialized disciplines of immunology and virology.

The stimulus to this phenomenal advance was the establishment of the bacterial cause of infectious disease and with this elucidation came the introduction of exact diagnosis by etiology, of specific therapy and of preventive medicine, all founded on verifiable fact. The profound reformation of medical thought required by the new knowledge of bacteriology was only brought about by a bitter struggle against almost unbelievable opposition, but out of it arose the beginnings of experimental medicine. Thus, bacteriology did not become merely a useful helping hand but the guiding finger and wrought such changes in human health alone that, if it be a benefit, it must at least equal the contribution to human

welfare of any other branch of science. To achieve this it has changed and continues still to change the order of importance of various diseases as the cause of death in different age periods. Diseases such as typhoid fever, diphtheria and pneumonia, as examples, have been reduced from prevalence with a high mortality to almost a rarity. So too the scourge of epidemics has been changed to a threat of danger manifest only if the required precautions are neglected and the fatal menace of some diseases has been softened. Meanwhile, in the course of the lives of people living today, the marvellous achievement of modern surgery was made possible by lifting it out of the despond of "laudable pus" into the security of asepsis. These are only general instances to give point to greater hope for the future.

Through the ages humanity, of all races, used the products of fermentation in various forms of food and drink or for the making of desirable utilities, without an inkling of the processes involved. The relation of the causes of fermentation and of infectious disease was hardly even suspected for some two centuries after bacteria were first definitely seen and figured by Leeuwenhoek (1676), and, though he did describe them in pus, his discoveries stirred interest

\* The names and dates given relate to the initiation of major trends or developments of medical bacteriology. W. Bulloch's *History of Bacteriology* (Oxford University Press, 1938) can be consulted with advantage.

in heterogenesis rather than disease. It was of course recognized that certain diseases were catching and that some conferred immunity from a second attack, but the idea of contagion passing from one individual to another, made evident by common observation in plague and syphilis, was completely missed in many another disease and was almost certainly exaggerated in leprosy. Fracastoro in his book on contagion (1546) was probably the first to indicate that "infection itself is composed of minute and insensible particles and proceeds from them" and he wondered whether all contagion may not be a putrefaction; he recognized that "the infection is the same for him who has received or has given the infection: also we speak of infection when the same virus has touched one or the other."

Much can be read into the early speculative writings, and it is well to be cautious in interpreting them, for in most instances it seems evident that the words and phrases used should not be accepted strictly in their modern meaning. Kircher (1658), probably the first to make direct microscopic studies of disease, examined putrifying materials and even blood from plague patients to postulate animated corpuscles which constitute the effluvia and scatter new seeds of contagion. He supposed a tenacity of life in them and that it is difficult to wash them away, so he recommended burning in the fire clothing and household goods infected with the contagion. The immutable specificity of contagious diseases was indicated by the practice of "variolation," but it was forcefully expressed by Thomas Fuller (1654-1734) who said that one could not change into another "any more than a Hen can breed a Duck," and he emphasized it further by saying "consequently one Sort cannot be a Preservative against any other Sort."

Speculation on what might have happened is futile, but Spallanzani (1775) seems to have only barely failed from re-

vealing the science of bacteriology in the course of his efforts to disprove heterogenesis as upheld by Needham. Spallanzani recognized and grew bacteria in sterilized media, he discovered forms which grew when deprived of air and he discovered "germs," which we now call endospores, of a greater resistance to heat than the forms they gave rise to. By his rigid maintenance of conditions of experiment for his intention, he failed to inoculate his media selectively on purpose and to realize the full general significance of his discoveries. It had all to be rediscovered nearly a century later by Pasteur, who also took pains during his studies on fermentation to disprove heterogenesis as maintained by Bastian and by Pouchet without losing sight of the singularly far-reaching importance of his discoveries.

Meanwhile the empirical method of probing the cause of infectious disease proceeded, with a diversity of observations and experiment too extensive to enumerate. Outstanding among these, because of their subsequent influence, are John Hunter's ill-fated self-inoculation with syphilis (1767) from a case of gonorrhea and Jenner's introduction of vaccination (1796) against smallpox, using material from naturally acquired cowpox. Hunter's experiment was a grave misfortune to himself, and, because he did not recognize a mixed infection, the belief persisted for very many years that syphilis and gonorrhoea were the same disease: a warning we cannot ignore today. Jenner's triumphant success, in substantiating a popular belief, resulted in widespread vaccination which reduced an almost world-wide disease to insignificance. The fatal propensity of smallpox is not better illustrated than by its ravages amongst the Indians of Canada and the United States in 1780, and 1869 and 1870, when whole tribes perished and others were decimated; these epidemics also provide strong evidence of the efficacy of vaccination when properly done and of its failure when care is insufficient. Of the same order of general impor-



tance were the insistence of Holmes (1843) and Semmelweis (1847-1849) on practical methods and cleanliness, for the prevention of puerperal sepsis and of blood poisoning from putrid wounds.

Improvements in the microscope led to more definite discoveries, and micro-organisms became associated with disease processes. The cause of favus found by Schönlein (1839) was used by Remak (1842) to reproduce the disease. Bassi (1837), from his work on disease of silkworms, prophesied that microscopic organisms would be found the cause of human disease, and similar suggestions came from many others. Henle (1840), in making the same prediction, drew up a statement of the conditions which would have to be satisfied to provide proof of a causal relationship. Similar postulates are ascribed to Robert Koch, but they do not appear in concise form in his writings. The first convincing discovery of microbial disease was the finding by Davaine (1850) of minute "infusoria" in the blood of sheep which had died of anthrax. Stimulated by Pasteur he returned to this discovery in 1863 and published it finally in 1864. The disease was transferred by inoculation of healthy animals with blood containing the rods he had found, and inoculation remained effective even when the blood was diluted a million times. The subsequent work on anthrax by Koch (1876) and Pasteur (1877) is virtually the starting point of pathogenic bacteriology and was founded on the earlier work of Pasteur from 1857 onwards.

The beginnings of the science of bacteriology emanated from Pasteur's interest in fermentation, which was stirred by his discovery (1848) of the selective use of dextro-rotatory tartaric acid by a mould which neglected the levorotatory form. His studies of lactic acid fermentation (1857) and of alcoholic fermentation (1860) led him to the necessity of disproving the hypothesis of heterogenesis (1861) in order to substantiate his demonstration of specificity of fer-

ments. The making of wine in France at that time was encountering an enormous reduction in quantity and a deterioration in quality and keeping power, partly due to an *Oidium* disease of the vines and partly to *Phylloxera*. This brought Pasteur to study the "flower of wine" and the "flower of vinegar" (1862) and led to his study of the making, ripening and preserving of wines and beer (1863) and eventually his studies of putrefaction and anaerobiasis. Diseases of silkworms next claimed his attention (1865-1869), and the procedures he instituted not only saved the industry in France, but their wide adoption is still the practice; it is a perfect example of the detection of infected individuals and controlling the spread of disease by isolating them. The war of 1870 stimulated his studies of infected wounds, and he translated his ideas of specificity of fermentations into specificity of infections. From this grew Lister's work and the introduction of antiseptics with the eventual development of aseptic technic as the work of many subsequent investigators. Pasteur went on to work at anthrax (1877), developing his vaccines and proving their worth (1881), then chicken cholera (1880) and swine erysipelas (1882)—to all of which his genius insured phenomenal results in the recognition of causative agents and specific immunization. Pasteur achieved greatest fame by his work on rabies. Recognizing the site of infection, he obtained a source of vaccine, even though he could not isolate the organism, and he developed a process of attenuation of the virus as well as a procedure for its application which is still used widely and is not surpassed by more recent methods. Pasteur is very rightly claimed to be the father of bacteriology.

Robert Koch, who started his work just when Pasteur had initiated the concept of specificity of infectious disease, contributed enormously and most particularly by his developments of bacteriologic technic. In his studies of anthrax (1876) Koch isolated

the bacillus in pure culture and established its infectivity. This was the first purposive isolation of a pure culture. He proceeded then to study traumatic infectious disease (1877) and developed the technic of isolation of pathogenic bacteria in pure culture from mixtures (1878-1881) so effectively that his methods are largely used today. He also used the newly discovered aniline dyes to great advantage in demonstrating bacteria microscopically. After Villemin (1865) had shown that tuberculosis, of both man and animals, could be transmitted by inoculation from man to animals and from one animal to another, Koch (1882) discovered the causative organism of tuberculosis. Later the differentiation into human type and bovine type was done by Theobald Smith (1896), and Rivolta (1889) and Maffucci (1890) discovered the related organism of avian tuberculosis. Koch went on to the discovery of the cholera vibrio (1883) making important contributions to the knowledge of that devastating disease, but his discovery of tuberculin (1890) was temporarily detracted from by the claim of its being a cure. The greatest of his many contributions was the discovery of methods of isolation and study of bacteria in pure culture and the procedure by which to study their infectivity.

Largely as the result of the work of Pasteur and Koch the isolation and identification of causative organisms of disease by many investigators proceeded apace. Long lists could be made of pathogenic microorganisms with the date of discovery and even longer lists of bacteria important or unimportant to other human interests. These dates and authors can be found in manuals of determinative bacteriology, and the exciting history of their discovery is in the original papers or special chapters of books.

The isolation of the diphtheria bacillus by Letzerich and Klebs (1881) and by Klebs and Loeffler (1883-1884) and of the tetanus bacillus by Kitasato (1889), after

it was seen by Nicolaier (1884), opened the way for one of the most important chapters in bacteriology, the discovery of toxin and antitoxin. Loeffler (1887) supposed the production of a poison to explain the results of his inoculation experiments using cultures of the diphtheria bacillus and Roux and Yersin (1888) demonstrated the toxin in filtrates of cultures. This was followed by Knud Faber (1890) showing that the tetanus bacillus also secreted a toxin. These various discoveries stimulated tremendous work on diphtheria resulting in the discovery of antitoxin by Behring (1890) for diphtheria and by Behring and Kitasato (1890) for tetanus. The brilliant confirmation of this by Roux and Martin (1894), who first immunized horses, and gave notoriety to serum therapy, resulted in the spectacular drop in the mortality rate of diphtheria. The first serum treatment in man was instigated by Behring and Wernicke and actually was done on Dec. 25, 1891. Ehrlich (1896) introduced standardization of toxin and antitoxin, thereby contributing greatly to their successful use in the treatment of disease and to the knowledge of their working.

In recent years, through the work of Ramon (1925), active immunization with formalin-treated diphtheria toxin (Anatoxine or Toxoid) has all but eliminated, where it is used, the incidence of diphtheria in children. Before this, immunization had a measure of success using toxin-antitoxin mixtures which Babes (1895) had proved on guinea pigs and Behring (1913) first used on humans. It was most effectively forwarded by Park (1913-1918) and controlled by the intradermal test of immunity introduced by Schick (1913) on the basis of the intracutaneous test used in animals by Roemer (1909). But the immediate influence of the early discoveries was to over-emphasize the possibilities and hopes of humoral immunity, and toxins and antitoxins were sought for everything, often in vain. However, this search and interest oc-



casioned the mixing of cultures, filtrates of cultures and immune serum, and the frequently unforeseen results introduced entirely new procedures and concepts into medicine. Thus Buchner (1889) found complement (alexin), Fodor (1886) the bactericidal action of normal rabbit serum for anthrax bacilli, Nuttall (1888) the dependence of bactericidin on complement and Richard Pfeiffer (1894) showed that with cholera vibrios immunization greatly intensified bactericidal activity.

Between 1870 and 1877 independent observations by Hayem, Klebs, von Recklinghausen, Waldeyer, Koch and others suggested that the leucocytes in pus, in which bacteria could be seen, were a suitable lodgement or a site of predilection for the microbes. Metchnikoff (1883) from a study of the activity of the amoeboid (mesodermal) cells of invertebrates and vertebrates, called them phagocytes and ascribed to them a protective activity by virtue of their destruction of ingested microbes. He conceived these scavenging microphages and macrophages to be the principal defence mechanism against infection, and a polemic arose and lasted several years between the Cellular Defence protagonists and those for Humoral Defence. Experiments to prove or disprove either hypothesis resulted in many interesting observations, and the recognition of immunity reactions and responses not suspected until then. Thus Denys and Leclef (1895) showed that immunization greatly increased phagocytosis and the work of Almroth Wright and his colleagues (1903) advanced knowledge of it and gave the name opsonin to this activity.

Charrin and Roger (1889) observed that *B. pyocyaneus* grown in immune rabbit serum first lost its motility and then grew in agglomerated masses, contrasting with the diffuse growth of motile organisms in normal serum. This was confirmed by many others for several different organisms and Bordet showed that heating the immune

serum at 56° C. did not alter its effect. Durham (1896), Gruber (1896) showed that the clumping of bacteria by immune serum was specific to the kind of bacterium and Durham named the reaction *agglutination*. Their work attracted keen attention and was applied immediately by Widal, Grunbaum, Semple and others to diagnostic tests to recognize formed antibodies in patients reacting to known cultures, or to recognize unknown cultures by their reaction with known immune sera. Bordet (1898) extended the reaction to specific agglutination of foreign, red-blood corpuscles by immune serum, and Landsteiner (1900) used it for the recognition of blood groups and the investigation of the antigens of erythrocytes, a knowledge now of much importance in blood-transfusion methods. Castellani (1902) devised the *absorption of agglutinin* test which ultimately led to the methods of antigenic analysis by purified absorbed sera and to the present trend to definition of bacterial species by their antigenic structure as illustrated by the genus *Salmonella*.

In investigating agglutination of erythrocytes by immune serum, Bordet (1898-1899) observed the lysis of the cells and established the relation of this lytic reaction to complement, showing that in the absence of complement simple agglutination occurred. He demonstrated the strict specificity of the reaction and showed that the complement was removed from the system in the process. Bordet and Gengou (1901) and Gengou (1902) used this immune haemolytic system as a delicate means of detecting the presence or absence of free complement. They showed that any immunity reaction taking place in the presence of complement removed or fixed the complement and thus they devised a most delicate test for the detection of interaction between antibody and antigen. This prompted many workers to apply the test to all sorts of purposes; the most widely known of these is the application of it by



Wassermann, Neisser and Bruck (1906) to the serodiagnosis of syphilis.

When mixing the filtrate of a cholera culture with its corresponding antiserum, Rudolph Kraus (1897) observed that a precipitate formed rapidly in the mixture and soon aggregated into flocculi which settled to the bottom of the tube. Identical results were obtained with typhoid and plague bacilli, but the reaction was strictly specific to the kind of bacterium. This observation was abundantly confirmed and quickly extended by Tchistovitch (1899) to eel serum, by Myers (1900) to egg albumen and by others to proteins of every source and to bacterial polysaccharides. It quickly had diagnostic applications in disease, and Nuttall (1902-1904) applied it to indicating phylogenetic relationship between mammalian species. It was applied to the identification of origin of blood stains (human, sheep, etc.), the sources of blood-sucking insect meals and of the adulteration of foods. It has had a most important application in the investigation of the mechanism of immunity reactions because it allows close quantitative determinations.

Out of this welter of new discoveries, one arising out of another and pointing in all directions, fiercely contended polemics arose and conflicting theories were elaborated to explain the situation. The theory of immunity propounded by Ehrlich (1896-1899, 1903, 1910) and known as "the side chain theory" primarily depended on the assumption of chemical affinity between antibody and antigen, with adjuvant factors added for special purposes. It did much to coordinate ideas and, above all, it afforded workers a terminology by means of which they could express themselves to be clearly understood. If for no other reason than this, Ehrlich's theory was a tremendous factor in the advances made in immunology in its early days. Ehrlich also made lasting contributions to experimental immunology, to chemotherapy and to the standardization

(1896) of diphtheria antitoxin. He distinguished active and passive immunity and characterized toxin. However, some observations were difficult to explain without making extensive new assumptions, and Bordet (1898 and 1909) introduced a physicochemical theory depending on a specific union of antibody and antigen, with the possibility of a chemical basis determining a specific adsorption, followed by a nonspecific action of electrolytes and a shift of electrical charge to result in the final manifestation of the reaction. This, largely replacing Ehrlich's hypothesis, stimulated a lot of exacting work over a period of years, and became elaborated to the view that antibody is specifically adsorbed, forming a partial or complete surface film. This theory too fails to fit all situations discoverable in immunity. Nor were these situations explained by the theory enunciated by Arrhenius and Madsen (1907) invoking a reversible reaction, analogous to that of weak acids and bases, and supposing the possibility of coexistence of free antibody, free antigen and antigen-antibody complex. The newest theory is that of Heidelberger (1935). This developed out of an extension of the work of Dean and Webb (1926) which showed antibody and antigen to react in terms of relative proportions independent of concentration. At the point of reference of optimal proportions there is no free antibody and no free antigen; this they regard as the *equivalent ratio*. Heidelberger introduced more delicate methods of determining quantitative reaction and measured and defined the action taking place more exactly. Heidelberger considers the reaction as the resultant of competing bimolecular reactions and the composition of the complex depends on the relative proportions in which the components meet rather than the concentration. The situation can be expressed by *mass-action* equations. This theory and Heidelberger's technique have stimulated most important work on a variety of problems including anti-

genic structure and specificity. Thus, there is a return to the importance of chemical constitution as the determining factor in immunity.

The application of agglutination and absorption of agglutinin reactions to the identification of bacteria resulted in the recognition of multiple antigenic components in the cells. This found expression in the definition of *types* within the species, the first of which was related by Gordon and Murray (1915) to the meningococcus, and others have since applied it to many other species, notably the pneumococcus and streptococcus. The definition of *type* and of *group antigens* in *Pneumococcus* by Avery and in *Streptococcus* by Lancefield (1928-1933) proved of great value and also indicated a relation between antigenic significance and chemical character. The importance of types was at first confined to directing specific serum therapy and the tracing of epidemiologic information, both of which led to an expansion of interest and an emphasis on immunological character in the exact identification of bacteria. The method was refined by the purification of sera, by specific absorption, to allow prompt and certain identification of antigenic components. When this method was applied intensively, while taking into account the flagellar and somatic antigens first discovered by Theobald Smith and Reagh (1903), the rough and smooth characters described by Arkwright (1921), the "H" and "O" conditions demonstrated by Weil and Felix (1920) and the diphasic variation discovered by Andrewes (1922), the complex distribution of the antigenic components in the variants of the species caused much confusion. Order was introduced in the case of *Salmonella* by Bruce-White (1934) and by Kaufmann (1934) so that, by their work and that of many others, strains in that genus can be defined by the antigens they exhibit.

Nevertheless, although there is an orderly arrangement by this method, it has

not yet allowed a satisfactory immunologic definition of bacterial genera and species.

Confusion still besets the immunologic definition of many other kinds of bacteria; others have not yet been subjected to it, so the accustomed criteria of morphology, growth characters and requirements, use of hydrolysable or fermentable substances loosely called "sugars," and the recognition of various by-products of metabolism, still have to be relied on for differentiation and identification of bacteria. These culture methods, which gave prodigious results, were made possible by four very simple technics: the introduction of the cotton plug by Schröder and von Dusch (1854); the sterilization of media by heat devised by Pasteur (1877), in part an application of the "marmite" of Papin (1681) and Koch (1881); the use of dyes by Weigert (1871, 1875) and elaborated by Koch (1877) and Ehrlich (1879); and the development of solid culture media by Koch (1881). The authorship of the use of "sugars" is obscure, but it should rank almost as highly as the above. Sternberg (1884) says that Pasteur took less account of structural characters than did Cohn, Nägeli, Dujardin and others and suggested that cultural requirements and special kinds of fermentation be relied on. At the same time Sternberg questions the value of "these species purely physiological." Before 1884 Mitscherlich observed that cane sugar, milk sugar, glycerine and even cellulose are assimilated. These were still the days when Cohn and others wrote with serious need on distinguishing "pseudobacteria." In the literature from 1900 onwards the descriptions of action on increasing numbers of "sugars" are common; they are tabulated in Matsushita's book (1901) and are prominent in other early textbooks. Special tubes for the appreciation of these fermentations were recommended by Theobald Smith and by Durham and are still in use.

As methods for the isolation and identification of bacteria improved, interest de-



veloped in their natural distribution and in the transmission of infection to cause disease. Case-to-case transmission, by contact and by fomites, was an expansion of the ideas of early speculative times and was substantiated by the instances of wound infection, puerperal sepsis, smallpox, etc. The extension of it to contamination of the environment, clothing and personal articles followed naturally. It was accentuated by the doctrine of "surgical fever" and the phlogenic and pyrogenic properties of pus started by Billroth (1860) and by the finding by Coze and Feltz (1866-1872) of "infuzoria" (either motile or in chains, name coined by O. F. Müller, 1773) in the blood and fluids of animals injected with putrid substances, which increased in lethal power in successive injections. Impetus, which carried final conviction, was given to the germ theory of disease by Klebs (1870-1873) investigating gunshot wounds, with consequent septicemia and pyemia, and the publication by Koch (1878) of his work on the "Aetiology of Traumatic Infective Disease." Koch's technical methods allowed Ogston (1880-1883) to associate suppuration and inflammation, spreading to septicemia and pyemia, with micrococci he named *Staphylococcus* and with Billroth's *Streptococcus* and led him to produce lesions in animals with cultures in eggs.

The recognition of contamination of water supplies and food through improper disposal of excrement brought about a new phase in the understanding of disease and led to legislation and control based upon bacteriologic knowledge. Snow (1849) first correlated epidemic cholera with contamination of water supplies and added confirmatory evidence during the epidemic of 1854. Although Budd (1856), still anticipating bacteriology, pointed out that typhoid is transmitted by the excreta of the patient, water-borne typhoid was not recognized until 1872, by Hagler in Switzerland, and milk was incriminated by Radcliffe and Power (1873) in the St. Marylebone outbreak of

enteric fever due to washing of utensils with polluted water. Flugge says in his textbook (1886) that pathogenic bacteria have never as yet been demonstrated with absolute certainty in water, but at that time the viability of typhoid bacilli and cholera vibrios in water was investigated by Bolton (1886) and a little later by Frankland, Chantemesse and others. It seems that the first isolation of typhoid bacilli from water was by Remlinger and Schneider (1896) and in the soil of infected barracks by Tryde and Salmonsén (1885); the first attempt at the sterilization of water mains was in the Maidstone epidemic of 1897, on the recommendation of Sims Woodhead, using large quantities of chloride of lime. The finding of typhoid bacilli in naturally contaminated water is still a difficult procedure and the recognition by Theobald Smith (1892) of the necessity of estimating the coliforms in water as an indication of faecal contamination proved an important step. The tracing of many notable outbreaks of typhoid, paratyphoid, cholera, food poisoning, diarrhoea, dysentery, scarlet fever, septic throats, infectious jaundice, et cetera, to contamination of water, milk and prepared foods has led to a great deal of special knowledge and to regulation and control. Sewage disposal, for the same reason, became principally concerned with elimination of contamination by pathogenic organisms and only secondarily with aesthetic and economic considerations.

Case-to-case transmission, even by indirect means of water, food and fomites, did not account for the incidence and distribution of many cases in epidemic cerebrospinal meningitis and failed to explain satisfactorily the source of some outbreaks of typhoid. The persistence of diphtheria bacilli in completely recovered cases was demonstrated by Roux, by Yersin and by Loeffler (1890), but their importance was not fully recognized. Albrecht and Ghon (1901) proved the presence of the menin-



gococcus in the nasopharynx, a finding notably expanded by von Lingelsheim (1906) to culminate eventually in the full elucidation of the role of healthy carriers by Gordon, Flack, Glover (1915-1917), and others. The investigation of the typhoid carrier dates from the statement by Koch (1902) that the typhoid patient or convalescent who happened to harbor the specific germ was the source of further infections. The bacteriologic examination of convalescents by Frosch, Drigalski, Donets (1903-1904), soon followed by others, proved the existence of faecal and urinary carriers. At the same time the existence of atypical ambulatory cases of enteric fever was recognized and an identical situation was soon found to prevail for paratyphoid and acute food poisoning (*Salmonella*). So the possibilities of the carrier condition was extensively investigated and obtained a prominent position as a source of preservation and dissemination of disease.

Among the diseases of antiquity, rabies, anthrax and tuberculosis were associated with animals, and the realization that they can be transmitted to man was delayed. It was known that rabies was transmitted by the bite of a mad dog, but man was thought immune until Celsus in the first century A.D. gave the name hydrophobia to the human disease, which was better described in the second century by Celsus Aurelianus. The paralytic form in man was recognized by van Swieten (1770), and Zinke (1804) demonstrated the infectivity of dog saliva to rabbits, dogs and chickens. Gruner (1813) recommended diagnostic inoculation of saliva from suspect dogs into test animals, and Magendie and Breschet (1821) identified human rabies with the animal disease by infecting a dog with saliva from a human case. This opened the way for Pasteur (1880) to study the transmission and control of rabies.

Devastating outbreaks of what appears to be anthrax of animals are found in the earliest writings. The disease was only rec-

ognized in man towards the end of the sixteenth century and the "black bain" was at times a scourge. Fournier (1769) described it as a disease of man and animals, and about this time Morgani, because of his opinions on infectious diseases, would not risk opening the chest of a wool comber suspected to have died of pulmonary anthrax. Human anthrax became recognized as a hazard of hide and hair handlers, and infection from shaving brush bristles imported from Siberia and China was proved (1915) by Elworthy.

Tuberculous lesions have been found in Egyptian mummies. The presence of tuberculous lesions in domestic animals gave rise to laws prohibiting their use as food. The lesions were subject to much confusion until the contagiousness of the disease was suspected by Rühling (1774), and animal tuberculosis was thought by Huzard (1790) to be identical with the human disease, though this view was not generally held. Koch's discovery of the tubercle bacillus (1882) led to the finding of it in cows' milk by St. Friis (1893) which was quickly confirmed and followed by Theobald Smith (1896) who differentiated the human and bovine varieties. The finding of the bovine tubercle bacillus in human cases was reported by Ravenel (1901), and its relation to tuberculosis, especially of children, was thereafter widely investigated by Park and Krumwiede (1910) and by A. S. Griffith (1914 onwards) who also first found it in cases of phthisis. It is also the bovine variety which Calmette and Guérin (1908) modified by growth in bile, to produce the B.C.G. strain used for vaccination (1921 onwards). The infectiousness of cattle tuberculosis to man urged the pasteurization of milk and the tuberculin testing of dairy herds, together with Government eradication schemes. Avian tuberculosis proved more an economic problem in poultry and swine; only a few cases have been reported in man.

Malta Fever, long known in man, was

brought into the category of animal diseases transmissible to man by the British Commission (1905-1907), by incriminating goats' milk; the disease has also been found in cows, sheep, mules and horses. Contagious abortion of cattle was suspected as infective for man after Schroeder and Cotten (1911) found *Brucella abortus* in milk and Larson and Sedgwick (1913) found antibodies in children, but the organism was first isolated by Duncan (1925) from a human case. *Brucella suis* is found in swine, horse, fowl and dog; it is chiefly American in distribution, and Keefer (1924) isolated it first from a human case.

Plague early made itself evident as a human disease but it is an open question whether the Philistine offering of mice and of "emerods" made of gold indicate recognition of its rodent origin. An 800-year-old sacred Hindu book, *Bhagavat Purana*, describes human and rat plague and recommends leaving houses where dead rats are found. Simond and Hankin (1898) suggested that plague was carried by fleas, and this was supported by Ashburton Thompson (1900) and Blackmore (1902) who did much to show the relation between rat epizootics and human plague, but the definitive association of plague with rodents and fleas dates to the report of the Indian Plague Research Commission (1906-1908). There was great scepticism of incriminating rats, and Lawson (1894), at the time Kitasato saw the plague bacillus and Yersin cultured and described it, writing of the Hongkong outbreak, said too much was being made of the infection of rats. Wu Lien-teh (1923) states that "references to rat mortality in the old European plague records are few and far between and lose much of their value by the inclusion of other animal species which we know now to be insusceptible." However, with the recognized importance of rodents other than rats, it is strange that Wu Lien-teh could find no earlier reference to tarbagan plague than 1895 which lends significance to the

observation that in California, where plague first appeared in 1900, the infection in wild rodents was not proved until 1908.

Tularemia, primarily a septicemic disease of rodents, was first proved in a human case by Vail, Wherry and Lamb (1914) and subsequently recognized and named by Francis (1920). Besides being acquired by the handling of infected animals (mainly jackrabbits), it is transmitted by blood-sucking flies and ticks. An interesting historical feature of this disease is that the recognition of its actual geographic distribution was so slow and bacteriology was so far advanced at the time of its discovery.

There are other important animal sources of human infection, among which is "food poisoning" due to members of the genus *Salmonella*. Most outbreaks of this have been due to prepared foods of animal flesh, and in Europe it has been associated with the slaughter of sick animals used for human food. The historical instance was the discovery of *Salmonella enteritidis* by Gärtner (1888), following the illness of 57 people who had eaten the meat of a sick cow. Sheep have been an especially common source of aertrycke infections in man, and, at the present time, dried egg powder has been shown to yield a number of different salmonellas.

With such discoveries as those of Manson (1877) on the transmission of *Filaria bancrofti* by the bite of *Culex fatigans*, of Bruce (1895) proving the transmission of 'Ngana, and later of Ross' work on *Proteosoma*, at a time when the bacteriologic cause of disease was itself a very exciting novelty, it is not surprising that the role of insects has been invoked as a possibility in bacterial infection from time to time. However, transmission of bacterial disease by blood-sucking arthropods has relatively few examples. Plague, for example, is transmitted by various species of rodent fleas, especially by the oriental rat flea (*Xenopsylla cheopis*) to man; the relapsing fevers by the louse (*Pediculus humanus*) and by



ticks (*Ornithodoros* of different species), varying strictly according to the species of spirochaete concerned; tularemia by deer-fly (*Chrysops discalis*), ticks (*Dermacentor andersoni*), rodent lice (*Haemodipsus ventricosus* and *Polyplax serratus*), a squirrel flea (*Ceratophyllus acutus*) and the stable fly (*Stomoxys calcitrans*), who has been blamed without justification for many crimes. Cases have occurred of possible transmission of anthrax and other diseases by the soiled proboscis of biting flies, but these have the character of accident.

The mention of "flies and disease" usually conjures up a vision of that teasing and restless insect with unpleasant habits, the housefly (*Musca domestica*), and there is no doubt of its complicity in the spread of typhoid, dysentery, cholera and food-poisoning salmonellas. It has been shown that flies are urgently attracted to fecal matter and decaying substances in which they lay their eggs or on which they feed. They have the habit of emptying their crop, wherever they may be, either to take up the material again or to leave it to eat afresh, and they defecate freely while feeding. The vomit spots and fecal spots of flies are found everywhere and the circumstances which favor spread of disease by flies vary partly with the climate but most with the prevailing sanitary conditions. This information was accumulated by intricate and difficult experiment and observation. Sydenham (1666) observed that an abundance of houseflies in the summer was succeeded by an unhealthy autumn, and Leidy (1864) attributed the spread of hospital gangrene to flies. Howard (1895) began the study of the bionomics of the housefly and proposed (1911) that it be called "typhoid fly" though admitting that the phrase was an overemphasis. Hewitt (1907-1912) made detailed studies of the anatomy of flies and believed the housefly plays an important part in the dissemination of disease "when the necessary conditions are present." To Howard and Hewitt great

credit is due for the work they did, mainly based on epidemiologic evidence, as well as the work they stimulated. A long series of experiments was carried out by Graham-Smith (1910 onwards) on the distribution of bacteria by nonbiting flies; he very carefully studied their feeding mechanisms, the functions of the crop and proventriculus, the process of regurgitation and defecation, as well as the habits of flies, especially before and after feeding. The range of flight of houseflies was first studied by Arnold (1907) and extended by Copeman, Howlett and Merriman (1911), Hewitt (1912) and others, giving the greatest range observed as 700 yards of actual flight. Sandwith (1904) described the habits of flies conducive to spread of disease, especially of ophthalmia in Egypt, and Bancroft (1769) speculated on the transmission of yaws by flies. Nichols (1912) believed a small fly (*Oscinus pallipes*) to be responsible for inoculating surface injuries and causing the majority of cases of yaws in the West Indies; he also demonstrated that wild flies carry fecal contamination. Hamilton (1903) was the first to isolate typhoid bacilli from wild flies caught in the vicinity of cases of typhoid in Chicago. Ficker (1903) did the same in Leipzig, and similar observations were soon made by many others, on various kinds of organisms and different species of flies. Extensive investigations were carried out by many of the above-mentioned observers, and by others, on the distribution and duration of the bacteria on and in the flies, and epidemiologic data of all kinds was collected. However, though abounding in interest, this important phase of bacteriology is still far from complete.

The need to grow bacteria in pure culture on prepared media, with the limitations set by bacterial morphology as revealed by microscopy, developed the method of differentiation by their physiologic processes. This led naturally to the discovery of special growth requirements



and biochemical activities and so by stages to the fruitful study of enzymes on the one hand and to the use, production and estimation of vitamins on the other. These are active lines of investigation which have not yet spent the impetus of the interest they inspire of themselves, nor have they outrun the promise of explaining the action of selective disinfectants, of the pathologic processes in some diseases and of benefits conferred by bacteria on other forms of life.

Lister's "ideal disinfectant" seemed on the verge of realization with the discovery of the sulfonamides. Landmarks in the field are the pioneer work of Ehrlich (1904-1915) on chemotherapy, especially in syphilis (1910), the clinical use of prontosil by Foerster (1933) and the communications by Domagk (1935). Colebrook and Kenny (1936) established the remarkable action of prontosil in streptococcus infections. This was followed (1938-1941) by the introduction of sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, etc., with safer and wider applications to Gram-positive organisms. Very quickly numerous synthetic products of the same general chemical nature were put on trial, and a frantic search was rampant for products acting on a wider range of bacteria, or regionally limited to intestinal infections, and, above all, with less toxicity to man. The benefits that these agents introduced in the treatment of war wounds and the freedom their use gave to the surgeon had a profound effect. The urgency of war increased the volume and rate of investigation to levels which never could have been realized in times of peace. The experiences in production and the realization of chemotherapeutic possibilities thus gained was instantly transferred to the astounding production of penicillin when its possibilities and advantages were demonstrated.

Incidental observations on synergy and antagonism between bacteria are scattered widely through the literature from quite early times, but usually there is little indi-

cation of more than a passing interest; thus, Garré (1887) and Freudenreich (1888) showed that media which had supported growth of one kind of bacterium might become antiseptic to another. On the basis of antagonism, Colebrook (1916) attempted to free meningococcus carriers by infecting their nasopharynx with pneumococcus. Rettger and Speary (1912) showed the bactericidal properties of egg white and Fleming (1921) made extensive studies of lysozyme in tears and in the secretions of mucous membranes. A sustained interest in this type of action led Fleming (1929) to the discovery of penicillin, which was developed by Florey (1941) to startling clinical applications. Dubos and Avery (1930) extracted from a cranberry bog bacillus an enzyme which digests off the capsule of pneumococcus Type III, rendering it avirulent. With this enzyme they could protect mice from infection and later they found specific enzymes to act on the capsule of other pneumococcus types. The efficacy of penicillin and its innocuity to man, has led to the persisting, stupendous, world-wide search for antibiotics from every possible source. A number have been described, but only streptomycin, discovered by Waksman (1940-1945), has so far proved of outstanding importance. Tyrothricin, discovered by Dubos (1940), has only limited applications. The complexity of the therapeutic applications and the need for proper control of antibiotics, has, if anything, enhanced the importance of the bacteriology laboratory. Furthermore, this actively growing phase of bacteriology has a very interesting bearing on the study of bacterial variation, the importance of which is still to be fully appreciated, including the development of drug-fast strains.

Haffkine (1896) described the self-sterilizing power of the water of the Jumna River, which was lost when the water was boiled, but the possibilities of this observation was not realized until the discoveries of Twort (1915) and D'Herelle (1917)

were appreciated. Twort described a lytic phenomenon and a vitreous appearance of cultures of staphylococcus derived from vaccinia lymph. The transforming agency could be transmitted to normal living cultures but failed to act on dead cultures; the active principle was filterable and somewhat heat-resistant. He formulated the several possibilities which might explain the phenomenon but did not insist on any one of them. D'Herelle rediscovered the same phenomenon, when working with the Shiga dysentery bacillus, extended the investigations considerably further to other bacteria, and even applied it to therapeutic purposes in man. He maintained that the effect was wrought by a living parasite of the bacterial cell and called it *bacteriophage*. The further history of bacteriophage belongs to the viruses, but their remarkable specificity to host requirements has been used to recognize race and type dif-

ferences within bacterial species by Evans (1934-1940) with streptococcus, by Craigie (1938-1942) with the typhoid bacillus, by Keogh (1938) with the diphtheria bacilli, and by Fisk (1942) with staphylococcus. The phage-typing of the typhoid bacillus has proved of epidemiologic importance in the hands of Helmer, Kerr, Dolman and Ranta (1939) and of Foley (1942) in tracing the source of outbreaks or individual cases. It has, too, a theoretical importance because the selectivity of the typhoid bacteriophage has been associated with the Vi antigen, which thus links it to problems of virulence and protective immunization. Furthermore, this most strange parasitism may well provide the solution of the mystery of the nature of viruses. Certainly the famous lines of Swift, inspired by Leeuwenhoek, finding a mite on the pupa of a flea, thus become even more appropriate.

## 2

# The Morphology and Physiology of Bacteria

### BACTERIAL MORPHOLOGY\*

#### SHAPE AND DIMENSIONS OF BACTERIA

Bacteria are microscopic unicellular organisms possessing dimensions of the order of low multiples or submultiples of  $\mu$ . They occur in only a few morphologic types (Figs. 1 and 2). The coccus forms are spherical or ellipsoidal. The rod or bacillary forms have one cellular axis markedly longer than either of the others and are commonly assumed to be cylindrical (for a contrary view, see Pijper 1946). The spirillar forms are helicoidal; the name vibrio (or comma) designates spirillar forms showing a single curve. Although the general morphologic pattern of each bacterial strain remains fairly constant under a given set of conditions, great variations may occur depending upon the past history of the culture, its age, and the environmental conditions under which growth takes place. Thus, streptococci and pneumococci can give rise to variant forms which are so elongated that they are more akin to the bacillary than to the coccus type. On the other hand, typical bacillary forms can occur in the coccoid state during certain phases of their growth cycle, or as

elongated myceliumlike filaments in the presence of certain toxic substances.

Much has been written concerning the existence of hypothetical, filterable and symplasmic phases of the life cycles of bacteria. However, positive knowledge is available only in the case of those forms of bacterial life which exhibit definite cellular organization. As in the case of other cells, the bacterial protoplasm is surrounded by a cytoplasmic membrane and by a more rigid cell wall. In other respects, however, the cellular organization of bacteria may differ from that of the classic animal and plant cells.

#### THE PROBLEM OF THE NUCLEUS

It has long been assumed that bacteria stand at the threshold of organized living matter and do not possess a nucleus. Many workers, on the other hand, believe that the transmission of hereditary characters in bacteria takes place through a nuclear mechanism analogous to that of higher plant and animal cells and that it involves the participation of chromosomes and genes. Even today one can find in the literature the most varied and apparently incompatible statements concerning the nuclear apparatus of bacteria. It has been

\* Detailed bibliographies concerning this topic have been assembled by Knaysi (1944) and Dubos (1945).



claimed that these organisms do not have nuclei, that their nuclear material is diffusely distributed throughout the protoplasm, that it is organized as an invisible gene string analogous to a single chromosome, that bacteria possess true vesicular nuclei, or that the whole bacterial body is a nucleus (Lewis, 1941). The minute dimensions of bacteria account in part for these conflicting views, as all intracellular bodies, and in particular the nucleus if it exists, are too close to the limit of resolution of visual microscopy to be readily studied. Even more important, perhaps, is the lack of adequate criteria to define the nucleus and to test for its presence. In higher organisms, the nucleus is a vesicular structure morphologically distinct from the cytoplasm, containing large amounts of nucleoprotein characterized in particular by desoxyribonucleic acid, undergoing a complex and characteristic process of division associated with cellular division, and acting as the bearer of hereditary characters. None of these criteria have been convincingly satisfied in the case of bacteria.

Bacteria exhibit great affinity for the basic nuclear dyes. Basophilia, however, is not a specific property of the nucleus and can be caused by different types of large molecular acidic substances. Among these, nucleic acids are of paramount importance, but ribonucleic acid is as effective as desoxyribonucleic acid in binding the basic dyes. Since the ribose type is very abundant in the cytoplasm, basophilia does not constitute sufficient evidence for the presence of nuclear material. Nucleic acids also exhibit intense absorption bands in ultra violet light; as in the case of basophilia, however, this property cannot be used for the differentiation of the ribose and desoxyribose compounds, because both absorb light in the same wave length. Identification of desoxyribonucleic acid in situ in the cells rests at the present time on two methods: (a) selective destruction of the ribose acid by the enzyme ribonuclease, (b) use of microchemical staining reactions which are more or less specific for the desoxyribose acid. The color test which is most commonly used for this purpose is the Feulgen reaction; under properly con-

trolled conditions the Feulgen reagent (reduced fuchsin) reacts with the aldehydic group of the desoxyribose sugar liberated from nucleic acid by acid hydrolysis.

When care is taken not to disrupt grossly the architectural organization of the cell, the Feulgen reaction, as well as other classic nuclear stains, reveal in many types of bacteria well-defined intracellular bodies which appear to undergo division concomitantly with the cell (Robinow, 1945). However, the Feulgen positive bodies are not evident in all bacterial species. Whether bacteria possess true nuclei remains therefore an open question, but a question which is not of only academic interest. As will be repeatedly emphasized, bacteria can undergo hereditary variation, affecting most of their characters, in particular those which condition pathogenicity. Moreover, the occurrence and transmission of hereditary changes in bacteria often suggest the participation of a gene mechanism similar to that operating in higher cells (Luria, 1947; Tatum and Lederberg, 1947). It is likely, therefore, that the understanding of the nature of bacterial variation depends upon more accurate knowledge of the structure of the nuclear apparatus in bacteria.

#### ENDOSPORES

Many species of bacteria give rise to resting forms known as endospores, so called because they are always produced within the mother cell, and which differ from resting forms in plant and animal species by their extreme resistance to heat and to toxic agents. Endospore formation is most commonly observed in the bacillary species, both aerobic (*Bacillus*) and anaerobic (*Clostridium*), and occurs only rarely among cocci and spirillar forms. Endospores may be spherical, ellipsoidal or cylindrical in shape, and terminal, central, or intermediate in position (Fig. 1 F, G, H and J). Although these morphologic characters are somewhat variable, their average manifes-

tations are sufficiently characteristic for each species to be of some use in taxonomy.

Spore formation involves migration and condensation of nuclear material at the locus where the spore is to form with subsequent development of a thick membrane. Only one spore is formed per mother cell in the majority of spore-bearing bacteria. The disporic state when it occurs is probably due to the fact that environmental conditions become favorable for spore formation at the time that the mother cell is about to divide. The mechanisms of the resistance of endospores to inimical influences is not understood. It is probable that the thickness and chemical nature of the spore membrane decrease its permeability to toxic agents and may be also responsible for the fact that endospores do not readily take the ordinary stains. These considerations, however, cannot account for resistance to prolonged heating and one is led to assume that the protoplasm of the spore is in a physicochemical condition which renders it unsusceptible to heat denaturation. It is worth mentioning in this respect that many ordinary enzymes which are universally pres-

ent in living protoplasm cannot be demonstrated in resting spores, although enzymatic activity appears concomitantly with spore germination, a time at which heat resistance also disappears.

It is often stated that sporulation is more likely to occur under conditions unsuitable for vegetative growth, whereas spore germination occurs when the environment again becomes favorable for cellular multiplication. In reality the factors involved in sporulation and germination are very poorly understood. Thus, in the case of anthrax, sporulation and multiplication of vegetative cells occur simultaneously in the same tissues. It is also known that sporulating species can mutate to give rise to variant strains which are unable to produce spores under any known environmental conditions. That exacting conditions are required for spore germination is shown by the failure of tetanus spores to develop in tissues until suitable reducing conditions are attained,

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FIG. 1. Morphologic types of bacteria. All but *G* are from culture in artificial media.

(A) Large coccus from human sputum. Impression smear; gelatin culture stained with fuchsin.

(B) Pneumococci. One-day-old culture, stained by Gram technic.

(C) Large sarcina (*Sarcina agilis*); living cells in hanging drop faintly stained with methylene blue.

(D) Diphtheria bacilli. Involution forms stained with methyl violet.

(E) Anthrax bacilli; gelatin culture stained with eosin methylene blue.

(F) Botulinus bacilli. Glucose gelatin culture showing endospores.

(G) Gas gangrene bacilli in guinea pig tissues, stained with methylene blue showing terminal spores.

(H) Tetanus bacilli showing terminal spores (drumsticks). Agar culture stained with fuchsin.

(J) Anthrax bacilli. Agar culture stained with fuchsin and showing central spores.

(K) Dysentery bacilli. Agar culture stained with fuchsin.

(L) Influenza bacilli. Culture stained with fuchsin.

(M) *Bacillus pyocyaneus*. Agar culture stained with fuchsin.

(N) Glanders bacilli. Potato culture stained with fuchsin.

(P) Plague bacilli. Culture on 3 per cent salt agar showing involution forms; stained with methylene blue.

(Q) Plague bacilli showing capsules. Culture 6 to 7 days old, with ferrotannate as mordant according to Loeffler, stained with fuchsin.

Unless otherwise stated, all reproductions are 1000 $\times$ . Details of the photographic technic are given in the source quoted below. The small numbers (in black) seen in the microphotographs are those appearing in Zettnow's Atlas. (Zettnow, E., Atlas Photographischer Tafeln nach Originalaufnahmen, in W. Kolle and A. Wassermann, Handbuch der pathogenen Mikroorganismen, Jena, Fischer, 1902.)



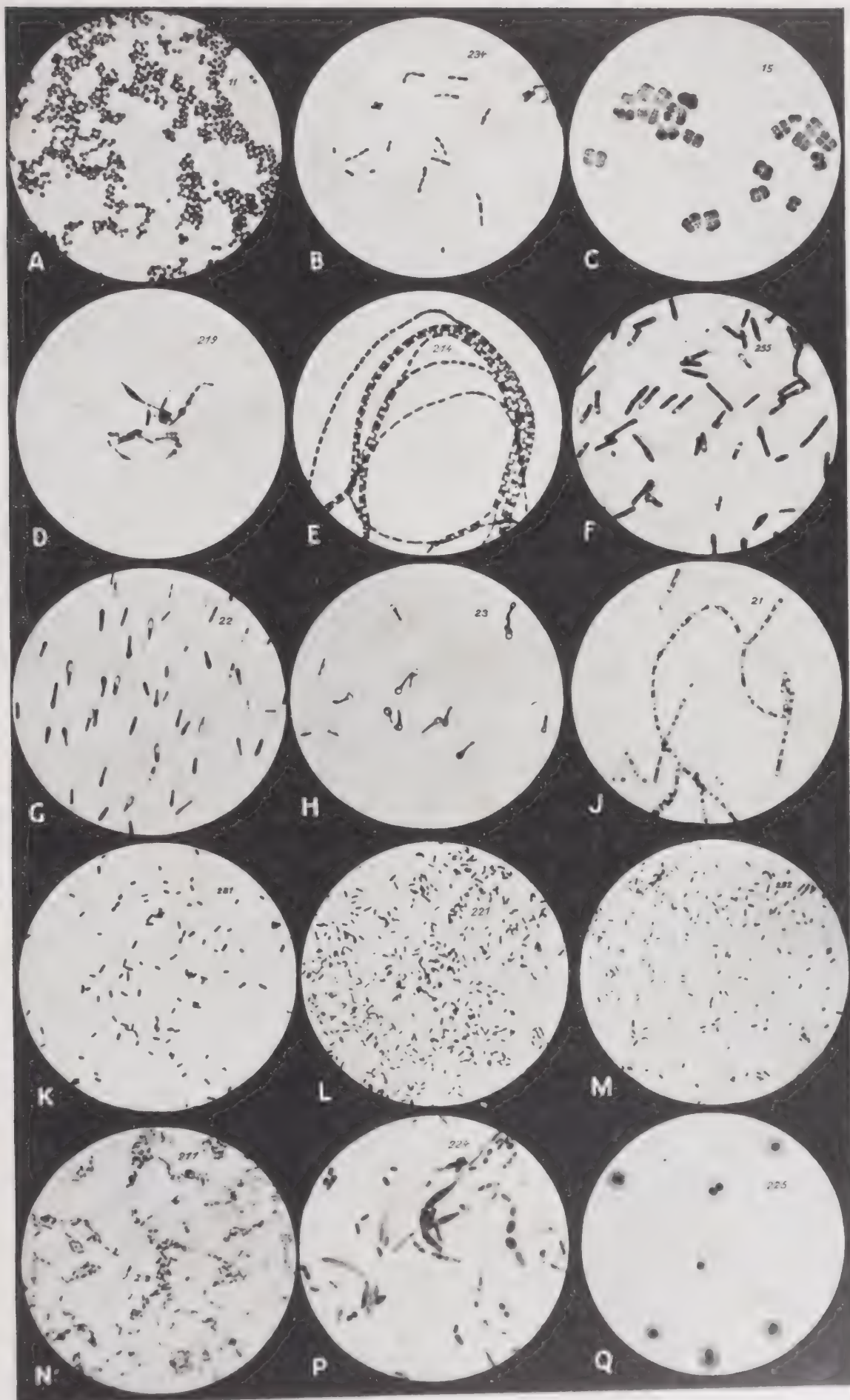


FIGURE 1



usually as a result of the coexistence of other bacterial contamination. Worthy of mention also is the fact that many spores exhibit the phenomenon of dormancy, i.e., they do not germinate immediately upon being transferred to a suitable environment, a fact of importance for the production in foods of botulinus toxin by *Clostridium botulinum*.

Endospores played an important part in the history of bacteriology by adding difficulties to the demonstration of the non-existence of spontaneous generation. It was only after F. Cohn and R. Koch had demonstrated the existence of heat-resistant resting forms produced by certain bacteria that one could explain the unexpected appearance of living micro-organisms in fluids in which all living forms had been presumably destroyed by boiling. The persistence for several years of the resistant endospores of *Bacillus anthracis* in soils previously contaminated by sick cattle also presented perplexing questions to the early students of the epidemiology of anthrax. The

danger arising from the survival of the spores of tetanus and other clostridia concerned in the contamination of wounds or food products adds practical significance to the interesting theoretical problems of the physicochemical factors which determine the resistance of these resting forms of bacteria.

#### OTHER INTRACELLULAR GRANULES

Examination of any microbial species by visible, ultraviolet or electronic microscopy reveals the existence of a variety of intracellular granules which have often been erroneously regarded as vesicular nuclei, endospores, or other reproductive bodies. The number, dimension, and distribution of these granules depend upon the age of the cell and upon its state of nutrition. They often decrease or even disappear under conditions of starvation and it is likely that many of them are storage material and perhaps in some cases products of elimination.

FIG. 2. Bacteria as they occur in infected tissues.

- (A) Streptococci in meningitis (spinal fluid), stained with fuchsin.
- (B) Pneumococci in exudate from human lung, stained with aniline water and fuchsin.
- (C) Ozena bacilli in human nasal secretion; very old smear stained with methylene azure.
- (D) Diphtheria bacilli in mucus from the trachea, stained with fuchsin.
- (E) Anthrax bacilli in mouse spleen, stained with fuchsin.
- (F) Leprosy bacilli. Smear from nasal mucus, stained by Ziehl-Nielsen. Note that the bacilli appear to be associated with the nucleus of the leukocytes.
- (G) Gonococci in pus, stained with methylene blue.
- (H) Meningococci. Section through the inner meninges stained with borax methylene blue. (500 $\times$ ).
- (J) Influenza bacilli in sputum, stained with fuchsin.
- (K) Mouse typhoid bacilli in mouse spleen, stained with fuchsin.
- (L) Coli bacilli in urine, stained with fuchsin.
- (M) Cholera stool showing predominantly cholera vibrios; a few large rods and cocci are also seen among the comma bacilli.
- (N) Fowl cholera bacilli in chicken blood stained with formol gentian violet.
- (P) Plague bacilli in rat spleen, stained with methylene blue.
- (Q) Relapsing fever spirochetes in human blood, stained with fuchsin.

Unless otherwise stated, all reproductions are 1000 $\times$ . Details of the photographic technique are given in the source quoted below. The small numbers (in black) seen in the microphotographs are those appearing in Zettnow's Atlas. (Zettnow, E., Atlas Photographischer Tafeln nach Originalaufnahmen, in W. Kolle and A. Wassermann, Handbuch der pathogenen Mikroorganismen, Jena, Fischer, 1902.)

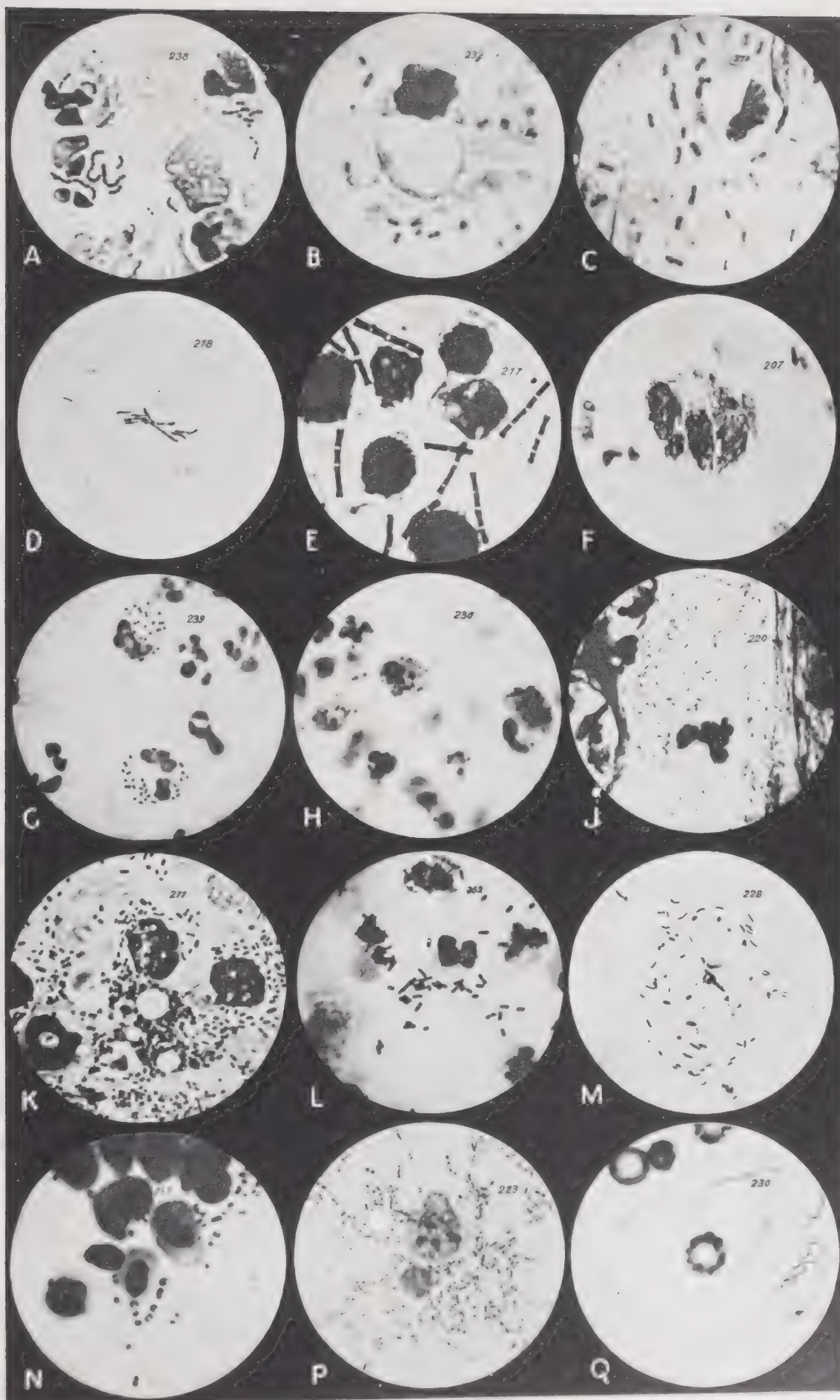


FIGURE 2



Granules of sulfur and of calcium carbonate have been identified in a number of saprophytic species. Fat droplets can be recognized in the living cell by their high-refractive power and can be stained by fat-soluble dyes. Carbohydrates related to dextrans, starch and glycogen can often be detected by treatment of the preparation with iodine. The bodies known under the name of Babes-Ernst granules, volutin granules, or metachromatic granules are widely distributed in bacteria. They contain ribonucleic acid and metaphosphate, substances which are probably responsible for the metachromatic staining given by these bodies in the presence of old solutions of methylene blue or toluidine blue.

Contrary to early views, there is no apparent relation between the number and size of these varied granules and the pathogenic properties of the cell. On the other hand, the relative frequency of occurrence of intracellular granules under given environmental conditions in certain bacterial species may be sufficiently characteristic to have some taxonomic value, in diphtheria bacilli, for example. It should be noted that many unjustified claims as to the existence in bacteria of gonidialike reproductive structures have been based on erroneous cytologic interpretations of the nature of the intracellular granules and vacuoles.

#### CELL MEMBRANES

Like other cells, all bacteria probably possess a cytoplasmic membrane which determines osmotic behavior and selective permeability properties. Because of its extreme thinness, this cytoplasmic membrane is not visible by direct microscopy, and there is no direct information concerning its chemical structure.

All bacteria appear to be surrounded by a cell wall which exhibits remarkable properties of rigidity, ductility and elasticity. Deep staining of the cell wall can be obtained only by the use of mordants, but there is usually no difficulty in distinguishing the structure by electron microscopy. Evidence of its existence is also afforded

by microdissection experiments and by the fact that the wall retains its form and position even after the cell has been plasmolyzed in hypertonic solutions. Despite many claims that cell walls contain cellulose, hemicellulose or chitin, there is no convincing knowledge of its chemical composition (Dubos, 1945). There is, however, indirect evidence, to be reviewed later, pointing to the association of Mg. ribonucleate, phospholipid-polysaccharide-peptide complexes, and mycolic acid, with the cell walls of, respectively, Gram-positive, Gram-negative, and acid-fast bacteria.

In addition to the cytoplasmic membrane and to the semirigid cell wall, many bacteria are surrounded by layers of high-molecular weight, colloidal material which varies in chemical composition in different groups and types of bacteria. Thus, many strains of streptococci produce under the proper cultural conditions a capsule consisting of hyaluronic acid; the capsules of virulent pneumococci consist of polysaccharidic acids the composition of which varies with each pneumococcus type; virulent strains of *Bacillus anthracis* are characterized by a capsule made up of a polypeptide of d-glutamic acid. In the case of several of the pneumococcus capsular polysaccharides and of hyaluronic acid, enzymes are known which can hydrolyse the capsular material as it occurs on the surface of the living bacteria. Interestingly enough, this process of enzymatic decapsulation does not affect the viability of the organisms concerned. As, in these cases at least, the capsule can be removed so readily without causing injury to its bearer, it appears possible, indeed likely, that the capsular material is not an essential structural constituent, but perhaps only a product of excretion which accumulates around the cell on account of its viscosity and limited diffusibility.

The classic bacterial capsules are readily detected by microscopic examination. Many bacteria, on the other hand, possess other



types of ectoplasmic layers which are apparently too thin to be demonstrable by microscopic cytologic methods even with the help of stains, but the existence of which can be surmised from indirect lines of evidence, in particular from the information provided by immunochemical reactions. For example, virulent streptococci of Group A possess on their surface protein constituents specific for each type of streptococcus (the so-called M proteins) which may be coexistent with the hyaluronic acid capsule mentioned above. Like hyaluronic acid, and the capsular polysaccharides of pneumococci, these M proteins can be destroyed by enzymatic hydrolysis without affecting the viability of the cells which bear them. Of special interest is the fact that M proteins can be destroyed by a cathepsin produced by the very same streptococcus which produces them and that failure to detect the presence of the protein may be due, not to failure of its production, but to the fact that hydrolytic destruction is too rapid to permit accumulation of detectable amounts (Elliott, 1945).

Other complex substances of ill-defined composition, often termed envelope antigens and to be described later as O and Vi antigens, have been shown to exist on the surface of certain forms of Gram-negative bacilli. It is most likely that other homologous surface constituents will be recognized in other bacteria when adequate methods become available for their detection.

Although the existence of different types of ectoplasmic layers has been illustrated in the preceding paragraphs by examples selected from among pathogenic microorganisms, analogous structures also exist in saprophytic species, and their existence is not therefore necessarily related to pathogenicity. Nevertheless, the ectoplasmic layers of pathogenic bacteria are of particular importance in medical bacteriology because it is through them that the invaded host comes into contact with the living parasite. As will be emphasized later, the

course and outcome of the infectious process depends in a large measure upon the qualitative and quantitative response of the infected host to these surface components of the parasite.

### FLAGELLA

A number of bacterial species are capable of independent motility. Creeping motion has been observed in a few of them, without any evidence of organs of locomotion. In the majority of free-swimming bacteria, however, motility is associated with the existence of flagella (Fig. 3, *top, left and right*). There is little convincing information concerning the nature, disposition and properties of these structures.

Flagella probably consist of extremely thin threads ( $0.02\ \mu$  to  $0.05\ \mu$ ) of protein nature, usually organized in fascicles. Their number and position are somewhat characteristic for each bacterial species; the adjectives monotrichate, amphitrichate, lophotrichate, and peritrichate, are used to define those which possess respectively one single flagellum at one pole of the cell; one flagellum at each pole; a tuft of flagella at one pole, or multiple flagella over their lateral surface. Bacterial motion is usually assumed to result from waves of contraction around the longitudinal axis of the flagella; however, it has been recently claimed that, far from being the cause of bacterial motility, flagella are in reality produced as a result of motion. According to this view, they are only artifacts resulting from the peeling off of shreds of the bacterial ectoplasm during motion, the latter being due to the flexibility of the whole bacterial body (Pijper, 1946; see also more recent discussion by Ørskov, 1947).

Whatever the real nature of flagella, there is no evidence that they play a significant part in the pathogenic behavior of bacteria, and antflagellar antibodies have never been found to exert a protective effect against infection. On the other hand, flagella are important for bacteriologic diagnosis. As already mentioned, their number and disposition on the cell is sufficiently characteristic to have some taxonomic value. More

important is the fact that the chemical nature of the flagellar material (known as the H antigen) varies sufficiently with each bacterial type to permit the development of specific diagnostic, immunologic reactions.

istic for each cell type. Many of these cellular constituents behave as antigens, i.e., they can elicit the production of serum antibodies when they are injected into experimental animals or during the course of

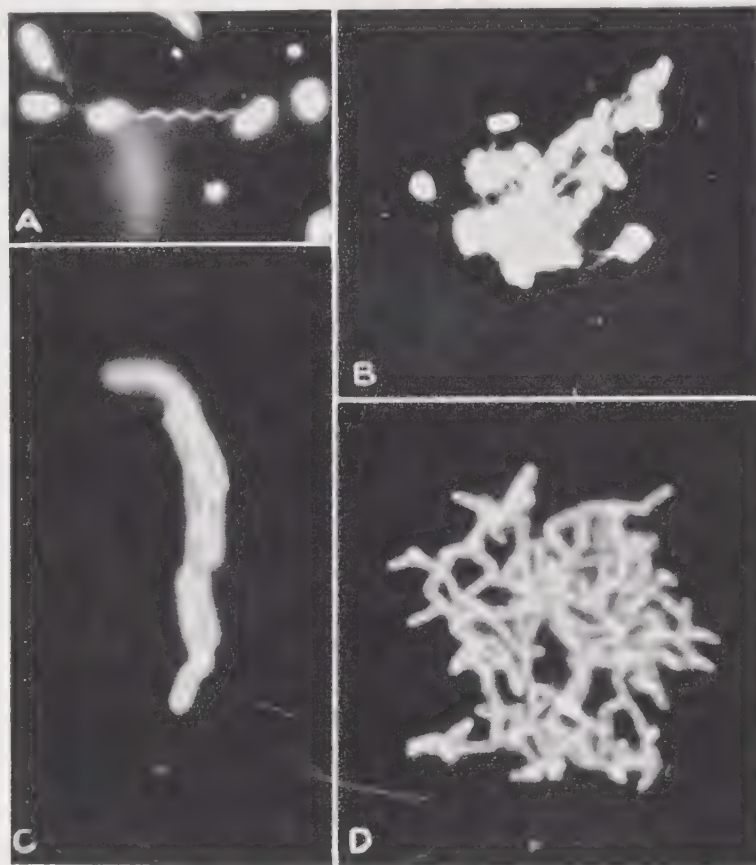


FIG. 3. Darkground studies of agglutination of *Salmonella typhi*. (A) Early stage of H-agglutination showing flagella becoming entangled. (B) Advanced H-agglutination showing loose clumps of bacilli. (C) Early stage of Vi agglutination. Note that bacilli adhere to each other along the longer cellular axis. (D) Completed O agglutination. Note end to end adherence of bacilli. (Dr. Adrianus Pijper, Pretoria, South Africa.)

Related bacterial species can be differentiated by means of antisera directed specifically against the flagellar (H) antigen of each species.

#### ANTIGENIC ANALYSIS OF CELLULAR STRUCTURE

Bacteria are made up of a multiplicity of different chemical constituents organized in the form of complex structures character

disease. These antibodies react selectively with the corresponding antigens; and can serve therefore as selective reagents for the detection of the latter. Immunologic reactions have been used, as we have already pointed out, for the recognition and identification of certain cellular constituents (for example, of some of the components of the ectoplasmic layers) which are not detectable by ordinary cytologic techniques. Antigenic analysis carried out by means of



immunochemical methods has greatly increased our knowledge of the organization of the bacterial cell; furthermore, this technic is of paramount importance for the identification of pathogenic bacteria, and for the understanding and control of the phenomena of immunity. It appears useful, therefore, to illustrate with a specific example, namely the typhoid bacillus, the relations of each specific antigen to its corresponding antibody (Fig. 3).

Typhoid bacilli directly isolated from pathological material possess a variety of constituents among which we shall mention: the flagellar material (H); 2 surface constituents (O and Vi), a number of ill-defined intracellular proteins which we shall group under the designation of Pr. It is possible to prepare fairly pure preparations of serum antibodies specific for each one of these constituents, and to test their effect against the whole bacterial cell. Living typhoid bacilli placed in anti Pr serum fail to show any reaction probably because the intracellular location of the Pr constituents prevents any contact with the extracellular antibody. On the contrary, living bacilli placed in anti H, or anti O, or anti Vi serum rapidly agglutinate as a result of the reaction of the antibodies with their corresponding antigens which are located on the cell surface. Moreover, the pattern of agglutination varies for each antigen-antibody system, suggesting that H, O, and Vi occupy different positions in the cell [Fig. 3] (Pijper, 1941).

As will be shown in subsequent chapters, these immunologic reactions are of paramount importance in the processes of antibacterial immunity.

#### REPRODUCTION IN BACTERIA

When grown under ordinary laboratory conditions, bacteria reproduce predominantly, if not exclusively, by binary fission.

The process begins probably by division of the cytoplasm while the new membrane grows inside the still intact mother cell. Constriction of the latter splits the new membrane into two and a cell wall is deposited on both sides of the membrane by the two daughter cells.

Many minor variations can greatly affect the appearance of the growth resulting from this process. Thus, if the cells fail to separate after formation of the membrane, they give rise to long chains of organisms connected by fine protoplasmic strands; the dividing wall may even remain incomplete despite division of the cytoplasm, resulting in the formation of long mycelium-like cells. There is indeed evidence that some long bacillary forms correspond in reality to a multiplicity of individuals in which the dividing membrane has failed to form although division of the nuclear material has taken place. Another factor which affects the general morphologic appearance of the growth resulting from binary fission is the plane in which division takes place. In the rod and spirillar forms the cell divides in a plane more or less perpendicular to the direction along which it grows. Among cocci, growth may occur in only one direction, giving rise to chains as in streptococci and pneumococci, or the direction may shift after each division with formation of cell aggregates (bunches and tetrads) as in the staphylococci and sarcina. Diphtheria and tubercle bacilli occasionally display rudimentary branching. Finally, the manner in which the cells separate depends upon post fission movements which result in different types of cellular arrangements (end to end, random, palisade).

Many claims have been made for the existence of more complex life cycles, involving reproduction by gonidialike bodies, amorphous symplasmic phases and primitive sexual processes. It is certain that most of these claims rest on faulty interpretations of cytologic observations, but a few are sufficiently substantiated to warrant brief mention here. One often finds, especially in old cultures, abnormal cells which are usually termed involution forms because they are assumed to represent degenerate or dying cells. Some of them, however, occur regularly in certain cultures and probably play a part in reproduction; the chromatin material of the cell aggregates in these "large swollen bodies" where it undergoes some rearrangement, then divides again to give rise to new bacilli (Dienes and Smith, 1944) (Fig. 4). It is too early to evaluate the significance of these recent ob-



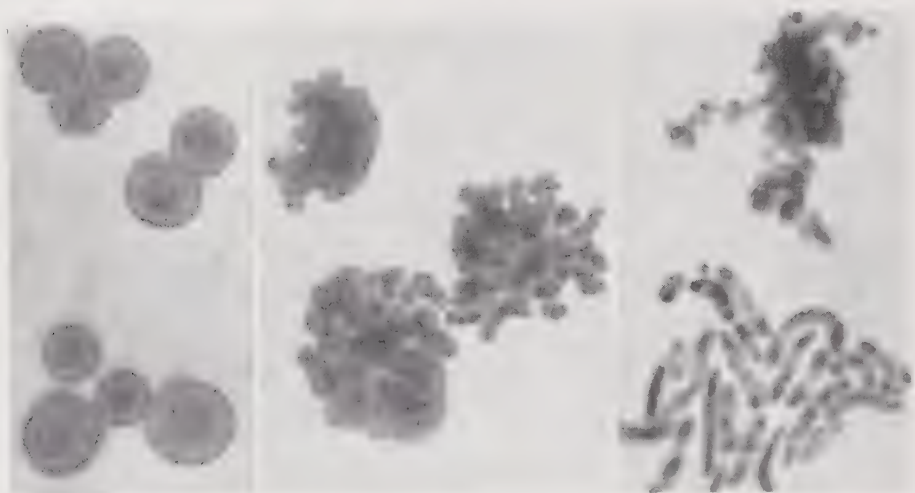


FIG. 4. Evidence for the transformation of large bodies into bacterial cells, as seen in a pleomorphic strain of *Bacteroides*. The culture was transferred to agar from broth media and stained at various times. Apparently the large bodies, left, segment and give rise to a group of rod forms (micro colonies). Alternatively, the large bodies may give rise to "L" colonies, in which the customary morphology of bacterial cells is absent; such an "L" type of colony is shown at the upper right. (Dienes, L., and Smith, W. E., 1944, The significance of pleomorphism in *Bacteroides* strains, *Journal of Bacteriology*, 48, 125-153.)

servations and to know whether they will throw light on the mechanism of bacterial variation.

## STAINING REACTIONS

### GENERAL STAINING CHARACTERISTICS

Many of the constituents of bacteria have their isoelectric point on the acid side of neutrality. This is true of certain cellular proteins as well as of the nucleic acids and phospholipids. In consequence, bacterial protoplasm exhibits great affinity for basic dyes at neutral or alkaline pH and is readily decolorized only by acid solutions. This general property has led to the development of a number of empirical and nonspecific staining techniques in which the basicity of the dye and the pH at which the staining procedure is carried out are important variables.

For certain particular purposes, the bacteriologist also uses other staining techniques based on better defined and more specific chemical reactions. We have already mentioned the Feulgen technic, which detects

the aldehyde group of the desoxyribose sugar in the nucleic acid of chromatin, and the staining of fat bodies with fat soluble dyes and with osmic acid. Several of the capsule staining technics make use of copper sulfate which precipitates and therefore fixes many capsular polysaccharides. In all these cases the staining technic goes beyond the mere detection of the cellular bodies and serves as a microchemical reaction which provides some knowledge concerning the chemical nature of these bodies.

On the other hand, there have been developed two empirical staining techniques which give highly characteristic results with certain groups of bacteria and which possess therefore great diagnostic value. They are the Gram and the acid-fast staining techniques.

### GRAM TECHNIC

The Gram stain consists of the following essential steps.

1. Bacteria are stained with a basic triphenylmethane dye (usually of the methyl-violet group).

2. They are mordanted by iodine in potassium iodide.

3. They are thoroughly washed with a neutral organic solvent (in general ethanol, or a mixture of ethanol and acetone).

4. They are counterstained with a dye of contrasting color (safranin, for example).

The bacterial species which are decolorized by alcohol following treatment with iodine and which take up the counterstain are referred to as Gram negative, whereas the Gram-positive species are those which retain the initial dye (crystal violet). Although the mechanism of the reaction is not yet understood, the following facts may have a bearing on it.

In general, the cells of the Gram-positive species appear to be more acidic than those of the Gram negative; when measured by a variety of technics, the "isoelectric point" of the former (expressing the overall charge of the different components of the cell) is lower than that of the latter. This finding is probably consistent with the recent discovery that the presence of magnesium ribonucleate in the bacterial cell is essential to the retention of the Gram stain. It is possible to treat the dead cells of Gram-positive organisms (yeast and bacteria) under such conditions that the extracted cells maintain their gross morphology but are no longer capable of retaining the basic dye when washed with alcohol. The fraction removed by the extraction procedure is rich in magnesium ribonucleate; moreover, addition of this salt to the extracted microbial cell restores to it the ability to retain the dye. This reaction exhibits a remarkable specificity. No other substance has been found so far to restore the staining characteristic, not even salts of desoxyribonucleic acid. Moreover, addition of Mg ribonucleate to normally Gram-negative cells does not convert them to the Gram-positive state. Finally, Mg ribonucleate cannot be extracted from Gram-negative bacteria by the technics which are successful with the Gram-positive species (Henry and Stacey, 1946).

#### ACID-FAST STAIN

Tubercle bacilli, and other mycobacteria, are difficult to stain by the ordinary dyes.

Staining is most readily achieved by treating these organisms with basic dyes in the presence of controlled concentrations of acid or alkali, preferably with the help of heating. Once stained under these conditions, tubercle bacilli retain the dye even when washed for prolonged periods of time with ethanol acidified with strong acid, a treatment which decolorizes all other bacterial types. On the basis of these observations, there have been developed a number of methods for the selective staining of acid-fast bacteria. The most commonly used of these is the Ziehl Neelsen technic, which involves the following steps.

The preparation is stained with a mixture of fuchsin (a basic dye) and carbolic acid. Although staining can take place slowly at room temperature, it is much hastened by heating (for example a few minutes at 100° C.). This procedure stains all kinds of bacteria, including endospores.

Thorough washing of the preparation with 95 per cent ethanol + 3 per cent mineral acid removes the dye from all bacteria except the mycobacteria (in particular, the pathogenic species); many types of spores also retain the fuchsin. For convenience of observation it is usual to counterstain the organisms decolorized by acid alcohol with a dye of a contrasting color.

Many theories invoking peculiar physicochemical properties of mycobacteria have been formulated to account for their staining properties. Thus, it is stated that carbolfuchsin is more soluble in the cell constituents of mycobacteria than in the decolorizing agent. It is of interest that in addition to mycobacteria, a few types of bacterial endospores and of animal cells (helminth eggs, hair) exhibit the property of acid fastness. Among constituents of mycobacteria, only one substance has been found to retain fuchsin when treated by the Ziehl Neelsen technic. It is mycolic acid, a complex acid alcohol which occurs in the form of an ester in the tubercle bacillus. Whether the presence of mycolic acid or like substances is sufficient to account for the acid fastness of mycobacteria remains, however, an unproven hypothesis.



Although the Gram and acid-fast staining reactions were developed as empirical procedures and their mechanisms remain obscure, it is interesting that they detect fundamental differences in the cellular structure of the different bacterial groups. As will be mentioned later, Gram-positive, Gram-negative and acid-fast bacteria differ not only in their staining properties but also in many of their most important characteristics.

## BACTERIAL METABOLISM \*

### AUTOTROPHY AND HETEROTROPHY

Comparative biochemical studies have shown that nutritional requirements, intermediary metabolism and synthetic processes are remarkably similar in all living cells and the bacteria prove no exception to this rule. It has long been customary to regard bacteria as exceedingly primitive organisms on the evolutionary scale. Indeed so-called autotrophic bacteria have been looked upon by many as the first form of life to make its appearance on this planet. Like green plants, the autotrophs can multiply in media containing only inorganic materials and carbon dioxide. As a source of energy for growth and synthesis of protoplasm they utilize energy released by oxidation of sulfur, iron, nitrite or even, in the case of purple bacteria, radiant energy from the sun. It is also true that the autotrophic bacteria are often remarkably resistant to the effect of their external environment and can withstand relatively large changes in external osmotic pressure, acidity, temperature and pressure. If, however, one examines the chemical structure of these so-called simple forms and the reactions by which they utilize energy, one finds the same complexity as in cells of the highest animals and plants (Umbreit, 1947). All bacterial cells contain a large number of

specific proteins, complex polysaccharides and lipids. Indeed almost all the enzymes—proteinases, flavoproteins, iron-enzymes, carboxylases, etc.—known to occur in the mammalian cell find their counterpart within the autotrophic bacterial cell.

The heterotrophic bacteria exhibit a decline in these extraordinary synthetic powers.\* The capacity to utilize energy from oxidation of inorganic materials is lost and the organism becomes dependent upon oxidation of carbohydrates or other organic substrates for its energy. Protein can no longer be synthesized from carbon dioxide and inorganic nitrogen and a requirement for one or many essential amino acids develops. Finally, specialized compounds, the vitamins, used for synthesis of certain enzymes, must be supplied from without. Many of the more fastidious pathogenic bacteria appear to possess fully as complex nutritive requirements as those of their hosts.

Bacteria are placed, therefore, at the bottom of the evolutionary scale because of their size and comparative simplicity of structure and not because they possess a primitive type of metabolism. It is not surprising, perhaps, that the chief biochemical characteristics present in multicellular organisms which have not, as yet, been found in bacteria are those concerned with special tissues such as nerve, muscle, the ductless glands, etc., and their interrelation. Thus, none of the hormones such as insulin, thyroxine or estrin have been found in bacteria nor have the fat-soluble vitamins A, D and E been detected.† These compounds are all concerned with the physiologic activity of multicellular organ-

\* General problems of bacterial metabolism are discussed in textbooks by Gale (1947) and by Stephenson (1948).

\* The point of view expressed here is that of Knight (1936) and Lwoff (1943). It should be mentioned that the opposite view is taken by Oparin (1938) and others who believe that organic materials were accidentally synthesized during the cooling of the earth's crust and that the first forms of life were heterotrophic. The autotrophs evolved by the gradual gain of synthetic powers.

† Both vitamins D and E are found in yeast, however.



isms as a whole and not with the existence of their individual cells.

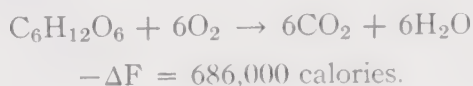
### ENERGY METABOLISM

The processes of growth and building of new protoplasm involve the synthesis by the cell, from simple substances of low energy content, of complex protein, nucleic acid, carbohydrate and lipid structures which are rich in energy. Although little is known of the mechanism of synthesis of these complex molecules by the cell, it is probable that most of the products are achieved by reactions involving dehydration or reduction, in other words by the reverse of hydrolysis and oxidation. Thus, most synthetic reactions require energy supplied from the outside. Energy may also be required to concentrate inorganic ions within the cell, to prevent free diffusion and mixing of the many reactive components of the cell, and for preservation of the semipermeable membrane. In general, the energy utilized for synthesis by heterotrophic organisms is obtained either by anaerobic oxidation (fermentation) or by aerobic oxidation (respiration) of suitable organic substrates, coupled with the formation of energy-rich phosphate bonds. The main energy source for bacterial growth is most often glucose or some carbohydrate readily converted to glucose, but may be fatty acids, alcohols, amino acids, etc.

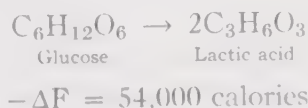
With respect to their dependence on oxygen supply, heterotrophic bacteria can be divided into three classes: the strict anaerobes, which are incapable of growth except under conditions of high reducing intensity (low oxidation-reduction potential); the aerobes which grow best under aerobic conditions; and the facultative anaerobes. The first class derive their energy exclusively from fermentation and possess neither the cytochrome system nor catalase. Aerobic bacteria, on the contrary, derive energy from respiration, that is, the complete oxidative breakdown of carbohydrate to carbon

dioxide and water, and contain the complete cytochrome system and catalase. According to older definitions, an aerobic organism was one which could grow only in the presence of oxygen and an anaerobe, one which could only multiply in the absence of oxygen. Facultative anaerobes were regarded as capable of growth under either aerobic or anaerobic conditions. These definitions no longer retain their original meaning as many bacterial species commonly classified as aerobic are capable of limited growth in the complete absence of oxygen. Moreover, the so-called strict anaerobes will flourish in liquid medium in air, provided a sufficiently low oxidation potential (high reducing intensity) is maintained. We shall thus consider as facultative anaerobes those organisms which obtain their energy primarily from anaerobic fermentation even when grown in air and which are incapable of true respiration, as, for example, most strains of streptococci. Other facultative anaerobes show somewhat increased growth in the presence of air and can utilize molecular oxygen to a limited extent. In all cases the cytochrome system is incomplete or lacking and it would seem preferable to consider those organisms which are incapable of true respiration as anaerobes at least from the point of view of their metabolism.

Most aerobic organisms under favorable conditions can break down glucose completely to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  according to the equation:



On the other hand, the anaerobes are capable of breaking down glucose only partially, as for example in the lactic acid fermentation:



Since the maximum energy available from the complete oxidation of glucose is 686 kg. cal. per mol., but only 54 kg. cal. per mol. from the lactic acid fermentation, it is clear that fermentation is a relatively wasteful and inefficient process as compared with respiration. It possesses the further disadvantage in laboratory cultivation of bacteria that large amounts of waste products, such as lactic acid and other acids, accumulate and soon cause cessation of growth unless neutralized or removed. The relative inefficiency of the fermentation process as compared with respiration is well illustrated by the maximum yields of diphtheria bacilli and of hemolytic streptococci which can be obtained through utilization of 1 Gm. of carbohydrate, in media containing an excess of other nutrients. Under aerobic conditions, the diphtheria bacillus breaks down carbohydrate almost completely to carbon dioxide and water yielding 0.5-0.6 Gm. bacteria per Gm. of carbohydrate. Hemolytic streptococci, on the other hand, growing under essentially anaerobic

conditions, yield only about 0.12 Gm. bacteria from each Gm. of glucose fermented to lactic acid.

#### FERMENTATION

According to Lipmann (1942) "Fermentations are energy-yielding rearrangements of the atoms constituting the glucose molecule. These are oxidation-reduction reactions in which, after cleavage, one part of the molecule is oxidized at the expense of the other which accordingly is reduced." As with cells of higher animals, muscle tissue for example, the first steps involve the anaerobic breakdown of glucose to pyruvic acid regardless of the final products and regardless of whether or not we are dealing with fermentation or respiration. The now classic Meyerhof-Embden scheme is summarized in Table 1 (Meyerhof, 1942; Barron, 1943).

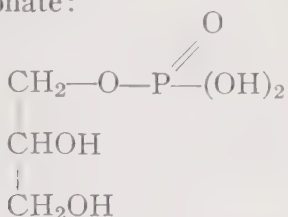
There are several points regarding the above scheme that merit discussion. In the first place it is important to note that all

TABLE 1. ANAEROBIC BREAKDOWN OF GLUCOSE ACCORDING TO MEYERHOF-EMBDEN SCHEME

REACTION	NET CHANGE IN ENERGY RICH PHOS- PHATE AS ATP
1. Glucose + ATP $\xrightarrow{\text{(hexokinase)}}$ glucose-6-phosphate + ADP	-1
2. Glucose-6-phosphate $\xrightleftharpoons{\text{(phosphohexoisomerase)}}$ fructose-6-phosphate	
3. Fructose-6-phosphate + ATP $\rightarrow$ fructose-1,6-diphosphate + ADP	-1
4. Fructose-1,6-diphosphate $\xrightleftharpoons{\text{(aldolase)}}$ 3-phosphoglyceraldehyde + dihydroxyacetonephosphate	
5. 3-Phosphoglyceraldehyde + H <sub>3</sub> PO <sub>4</sub> $\rightleftharpoons$ [1,3-diphosphoglyceraldehyde] $\xrightleftharpoons{\text{(isomerase)}}$ 1,3-diphosphoglyceric acid	
6. [1,3-Diphosphoglyceric acid] + DPN $\rightleftharpoons$ 1,3-diphosphoglyceric acid + DPNH <sub>2</sub>	
7. 1,3-Diphosphoglyceric acid + ADP $\rightleftharpoons$ 3-phosphoglyceric acid + ATP	+2
8. 3-Phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid	
9. 2-Phosphoglyceric acid $\rightleftharpoons$ phosphoenol pyruvic acid + H <sub>2</sub> O	
10. Phosphoenol pyruvic acid + ADP $\rightleftharpoons$ pyruvic acid + ATP	+2
11. Pyruvic acid + DPNH <sub>2</sub> $\xrightleftharpoons{\text{(lactic dehydrogenase)}}$ lactic acid + DPN	
Total	+2

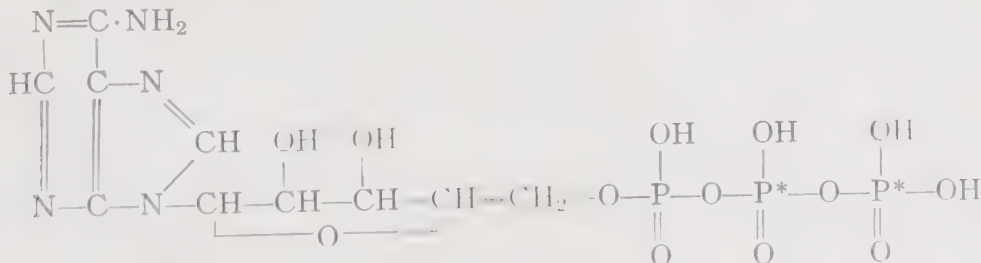
the steps in the chain except those involving phosphorylations with adenosine triphosphate (ATP) are completely reversible. This means that by putting energy back into the system, carbohydrate may be synthesized from pyruvic acid. It is also important to note that all the intermediate steps between glucose and pyruvic acid involve phosphate bonds. The breakdown of glucose cannot take place in the absence of phosphate. Phosphate bonds may be of two types discussed below (Lipmann, 1941).

1. A simple ester linkage may be formed by the reaction between phosphoric acid and an alcoholic hydroxyl group with elimination of water. This ester type of phosphate linkage is one of low energy content, and only 2,000 to 4,000 calories of free energy are available from its hydrolysis to alcohol and free acid. For example,  $\alpha$ -glycerol-phosphate:



and glucose-6-phosphate are esters of phosphoric acid which contain low energy phosphate bonds.

2. There are energy-rich phosphate bonds, on the other hand, which yield from 12,000 to 16,000 calories of energy on hydrolysis. These bonds may be of several kinds. Elimination of water between two molecules of phosphoric acid to form the anhydride yields an energy-rich linkage of the type found in adenosine-triphosphate (ATP):

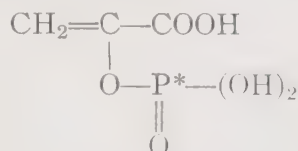


In ATP, the first phosphate is linked to ribose by a low-energy ester bond and the

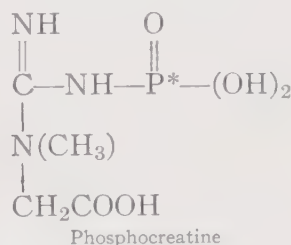
remaining two (marked with an asterisk) are energy-rich anhydride bonds. An energy-rich phosphate bond is also created by anhydride formation between a carboxyl group and phosphoric acid. 1,3-diphosphoglyceric acid contains one energy-rich bond of this type in addition to an ester phosphate linkage:



Energy-rich phosphate bonds may also be formed between acidic enolic hydroxyl groups and phosphoric acid by elimination of water as in 2-phosphoenol-pyruvic acid:



Finally, energy-rich phosphate bonds may be formed between nitrogen and phosphorus as in phosphoarginine and phosphocreatine (phosphagen):



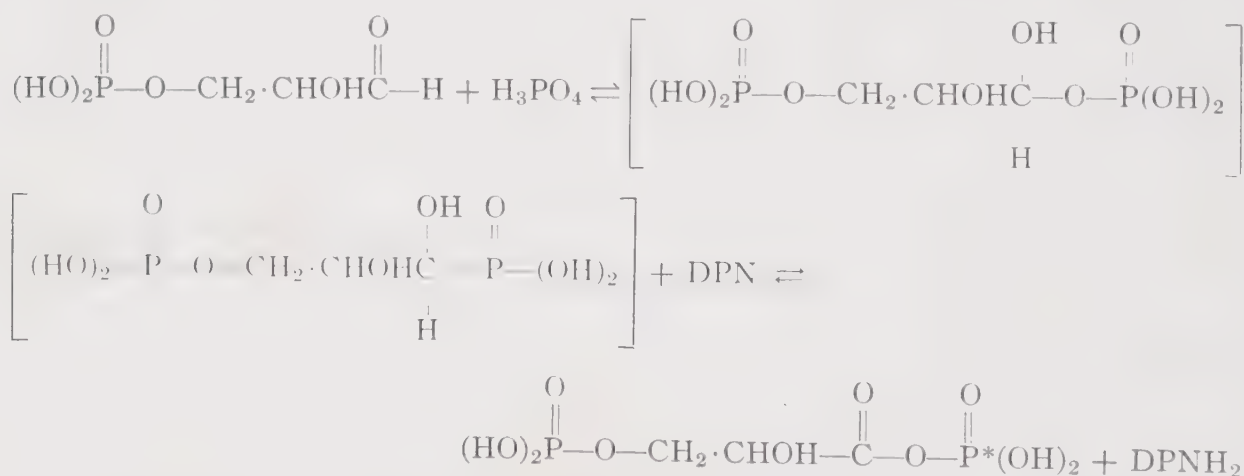
Phosphate bond energy in the form of adenosine-triphosphate (ATP) probably represents the main immediate source of energy for synthetic processes in the cell, for maintenance of internal osmotic pressure, etc. The exact manner by which phos-

phate bond energy is transferred to synthetic processes is still not clear, but prog-



ress is being made in this direction. ATP is a means for transfer of energy and probably not a means for storage. Energy is stored as carbohydrates such as glycogen or starch, fats and possibly nucleic acids and proteins.

Let us now return to a consideration of the Meyerhof-Embden scheme given in Table 1. The initial steps resulting in the formation of fructose-1,6-diphosphate from glucose involve the net loss of 2 energy-rich phosphate bonds from ATP to form 2 ester linkages. In step 5, 3-phosphoglycer-aldehyde reacts with inorganic phosphate to form 1,3-diphosphoglyceraldehyde.\* The coupling of step 5 with the step which follows may be regarded as the crucial reaction. The energy which would have been dissipated in the form of heat from the oxidation of the aldehyde to the acid is almost quantitatively converted to energy-rich phosphate bond energy:



As 2 molecules of the triose are oxidized per molecule of glucose, this results in the gain of 2 energy-rich phosphate bonds which are transferred to adenosine diphosphate (ADP) in the following step. Two more energy-rich phosphate bonds are formed by dehydration of 2-phosphoglyceric acid to phosphoenol-pyruvic acid and are transferred to ADP to form 2 additional

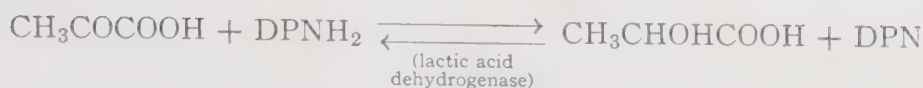
\* This compound has not been isolated in crystalline form. None the less it is difficult to postulate any other plausible mechanism for steps 5 and 6 except through 1,3-diphosphoglyceraldehyde.

molecules of ATP. Thus, the breakdown of 1 molecule of glucose to 2 molecules of pyruvate or lactate results in an overall gain of 2 energy-rich phosphate bonds (which are produced in the form of ATP) equivalent to 24,000 calories.

The above scheme now appears to be common to all types of living cells. The catalysts involved, such as adenosine triphosphate (ATP), and diphosphopyridine nucleotide (DPN, coenzyme I), have been found in all cells wherever they have been sought. Although most bacteria are able to synthesize these essential coenzymes, many parasitic and pathogenic species fail to grow unless nicotinic acid, adenine or, as in the case of *Hemophilus influenzae*, the complete DPN molecule is supplied to the culture medium.

Pyruvic acid is the key compound formed in the fermentation reactions. It is at this point that the pathways of fermentation

for different species or strains diverge. The central position occupied by pyruvic acid is illustrated in Table 2 in which some of the many reactions which this compound can undergo are listed. The particular type of fermentation predominating varies with each species of bacteria studied and often with different strains of a given species. Hemolytic streptococci and pneumococci, for example, produce lactic acid almost quantitatively from glucose through reduction of pyruvic acid:



The gonococcus, under anaerobic conditions, produces acetic acid in addition to lactic from pyruvic acid (Krebs, 1937):

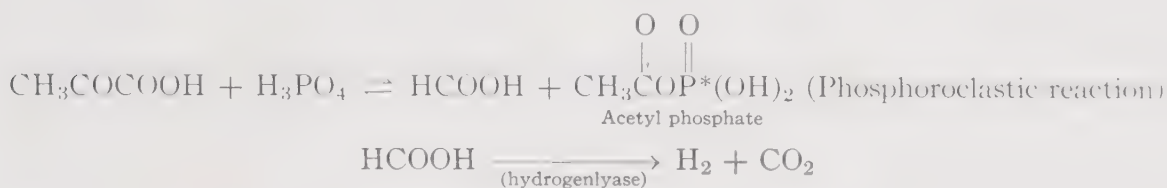


Other bacterial species may give high yields of propyl alcohol or propionic acid, ethyl alcohol, formic acid, butyl alcohol or

gen and carbon dioxide—a more efficient process resulting in increased growth.

Hydrogen is a frequent product of bac-

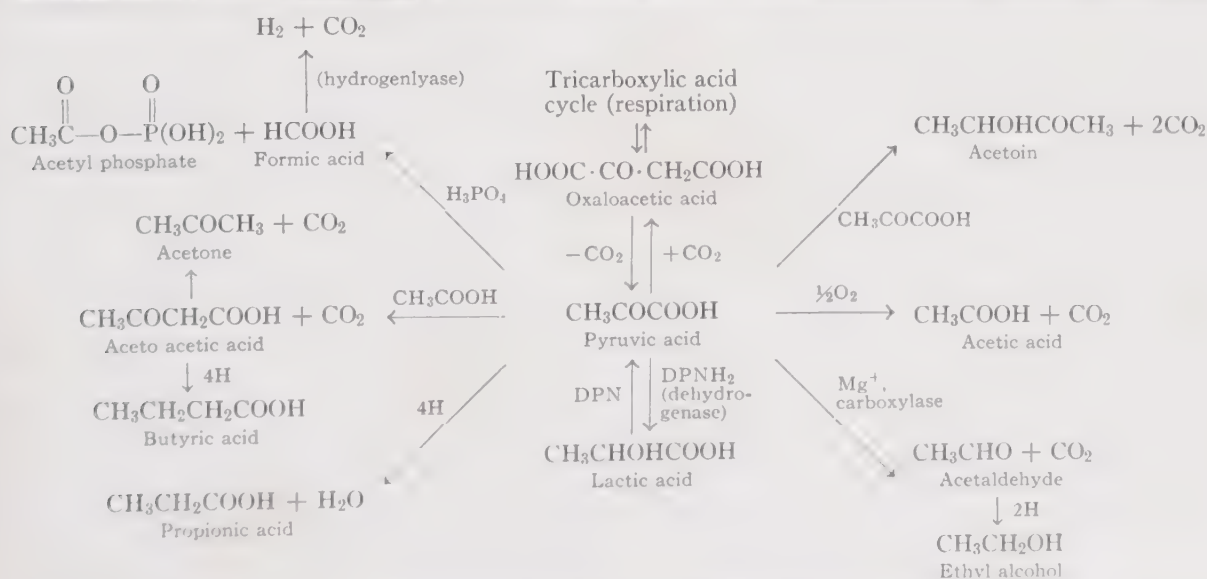
terial fermentation and is usually formed from formic acid by the enzyme, hydrogenlyase:



butyric acid—all derived from pyruvic acid. In many cases the pH or composition of the medium determines the nature of the final products from pyruvate. Thus *Cl. welchii* grown on a medium deficient in iron, undergoes a lactic-acid fermentation of glucose. At normal iron concentrations, however, the fermentation of glucose is predominantly to butyric and acetic acids with the production of large quantities of hydro-

It is probable that most of the end products of bacterial fermentation are also formed in mammalian tissues, but are not generally recognized since they are rapidly oxidized. Acetoacetic acid, precursor of butyric acid, and acetone are normal products of human intermediary metabolism which accumulate under pathologic conditions such as diabetes. Acetoin (acetyl-methyl carbinol), a product used to distin-

TABLE 2. SCHEME ILLUSTRATING THE CENTRAL POSITION OF PYRUVIC ACID IN FERMENTATION REACTIONS



guish certain soil bacteria from coliform bacilli in water analysis, is also produced by heart muscle enzymes.

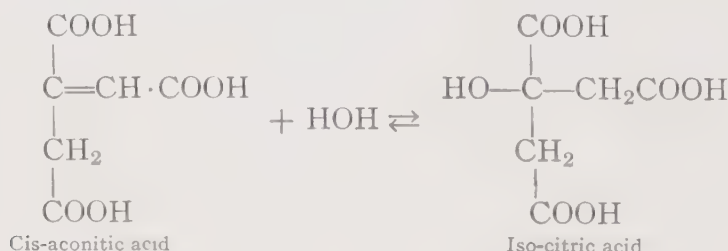
### RESPIRATION

It has already been pointed out that the complete oxidative breakdown of glucose



is a far more efficient means of obtaining energy for growth and synthesis than its partial anaerobic breakdown by fermentation. The initial breakdown of glucose to pyruvic acid is essential for both the respiration and fermentation processes and the steps followed are the same. Carbohydrate

trophic bacteria (particularly certain pathogenic species such as gonococci and brucella) require a high  $\text{CO}_2$  tension to initiate growth. Gladstone et al. (1935) had shown that many other bacteria fail to grow when the gaseous environment consists of  $\text{CO}_2$ -free air or nitrogen bubbled through the medium:



metabolism under aerobic conditions, however, is associated with diminished anaerobic glycolysis. This suppression of fermentation under aerobic conditions was first noted by Pasteur and is called the *Pasteur effect*.

In the respiration cycle, 4-carbon dicarboxylic acids and fixation of carbon dioxide by pyruvate play important roles (Ochoa, 1946). The assimilation of carbon dioxide by bacteria was first demonstrated by Wood and Werkman in 1936 from carbon balance studies on propionic acid bacteria and confirmed by them in 1940, using isotopic carbon (Wood et al., 1940). Soon afterwards carbon dioxide fixation by animal tissues was recognized. Prior to this discovery,  $\text{CO}_2$  assimilation had been regarded as an exclusive attribute of photosynthesis and of the autotrophic bacteria, although it had long been known that many hetero-

Although the equilibrium of this reaction lies far to the left, the reaction can proceed in the direction indicated because of the removal of oxaloacetate by reaction with pyruvate to yield  $\text{CO}_2$  and cis-aconitic acid. The latter then reacts with water to form isocitric acid:

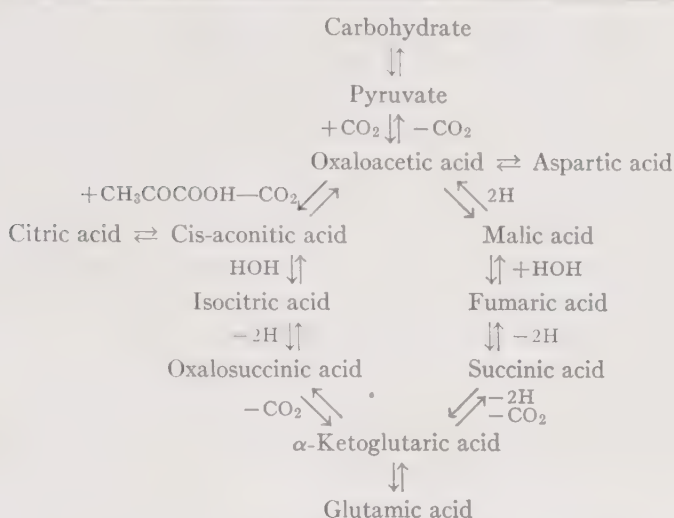
These are the first steps in the so-called tricarboxylic acid cycle of Krebs (1943) which is shown in Table 3.

By a series of reversible reactions, pyruvate derived from glucose is oxidized to carbon dioxide and water and oxaloacetate regenerated once more. So far as is known, the cycle is a universal means of aerobic oxidation by animals and plants. A few of its many implications will be noted here.

1. Carbon dioxide fixation by pyruvate is not the only means of entering the cycle. The oxidative deamination of glutamic acid, for example, can yield  $\alpha$ -ketoglutaric acid. A far more important means of obtaining this product occurs by transamination according to reactions discovered by Braunstein and Kritzmann (1937) who showed that tissue extracts catalyze the transfer of amino groups from aspartic and glutamic acids to certain keto-acids, notably

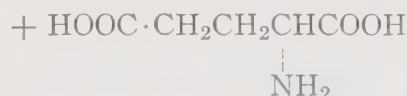


TABLE 3. THE TRICARBOXYLIC ACID CYCLE OF KREBS



$\alpha$ -ketoglutaric and pyruvic acids, with the formation of oxaloacetic and  $\alpha$ -ketoglutaric acids respectively:

reactions are reversible, the scheme can be used for carbohydrate synthesis (passing through the cycle in a clockwise direction)

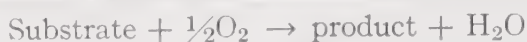
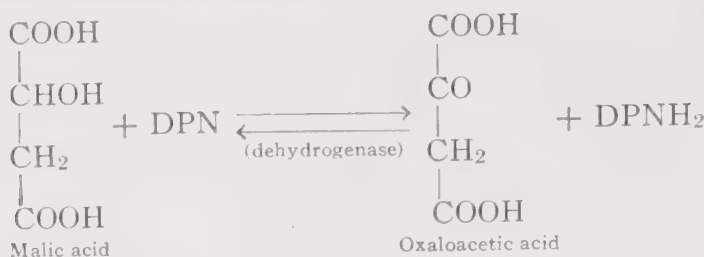


These reactions are catalysed by transaminases, enzymes which contain pyridoxal phosphate or pyridoxamine (vitamin B<sub>6</sub> derivatives) as prosthetic groups (Braunstein, 1947). It should be noted, however, that aspartic and glutamic acids are seldom found to be essential for nutrition of either bacteria or higher animals. It is not unlikely, therefore, that the cycle provides the usual means of synthesis of these two important amino acids.

2. All of the reactions in the cycle are reversible. The oxidation of one molecule of pyruvate by passing around the cycle in a counterclockwise direction results in a net gain of 266,000 calories of free energy equivalent to oxidation of half a glucose or one pyruvate molecule. Since all of the

provided energy is supplied from the outside as in the case of photosynthesis by green plants. Pyruvate is synthesized instead of being oxidized and is then transformed to carbohydrate through reversal of the Meyerhof-Embden scheme for anaerobic glycolysis.

3. The hydrogen atoms liberated by certain of the steps involving oxidation, i.e., oxidation of isocitrate to oxalosuccinate, and of malate back to oxaloacetate, are transferred to molecular oxygen through phosphopyridine nucleotides (DPN and TPN), flavoprotein and the cytochrome system. For example, malate is oxidized to oxaloacetate by DPN and the enzyme malic dehydrogenase:



It must be emphasized that this scheme is not always strictly followed; that in many cases one or more of the catalysts are bypassed during transfer of hydrogen to oxygen and that there probably exist intermediate steps involving cytochrome b and as yet unknown catalysts. (Keilin and Hartree 1939, 1940). None the less it is certain that in most instances cytochrome oxidase, cytochrome c and flavoprotein are involved.

According to our definition, all organisms capable of true respiration contain the cytochrome system. On the other hand the cytochromes are entirely absent from obligate anaerobes. The distribution of the cytochrome spectrum in various kinds of bacteria is illustrated in Table 4, adapted from Fujita and Kodama (1934). All organisms examined spectroscopically were grown on agar surfaces in air with the exception of the obligate anaerobes which were incubated anaerobically. It will be noted that organisms such as pneumococci and streptococci, even though capable of growth on a blood agar surface in the presence of air, lack both catalase and the cytochrome system. These organisms are generally classified as *facultative* anaerobes, although they most closely resemble the strict anaerobes

in their metabolism. Pneumococci and other facultative organisms of this type occupy an intermediate position between aerobes and strict anaerobes because they are capable of multiplication at a somewhat higher oxidation potential than the strict anaerobes and their growth is not inhibited by oxygen. Indeed pneumococci show some oxygen uptake in the presence of oxygen, probably due to flavoprotein. This cannot be regarded as true respiration, however, since acid products are incompletely oxidized.

Aerobic bacteria may possess very large amounts of catalase. *C. diphtheriae* and *Micrococcus lysodeikticus*, for example, contain approximately 1 or 2 per cent of catalase by dry weight. The catalase from the latter organism has recently been isolated in crystalline form and is some three times as active per unit weight as crystalline beef-liver catalase (Herbert, 1947). Since catalase is one of the most active enzymes known (one molecule will decompose 2,600,000 molecules of hydrogen peroxide per minute at 0° C.) it seems difficult to understand why such relatively large amounts are present in the cell if its sole function is to decompose hydrogen peroxide. It is probable that catalase promotes other re-

TABLE 4. DISTRIBUTION OF CYTOCHROMES AND OF CATALASE IN CERTAIN BACTERIA

SPECIES		CYTOCHROMES *	CATALASE †
Aerobes	Gram negative cocci.....	abc	+++
	<i>B. subtilis</i> .....	abc	++
	Baker's yeast.....	abc	+++
	<i>Ps. aeruginosa</i> (pyocyanea).....	<b>abc</b>	+++
	<i>M. tuberculosis</i> .....	<b>abc</b>	++
	<i>C. diphtheriae</i> .....	<b>abc</b>	++
	<i>Staphylococcus</i> .....	<b>abc</b>	++
	<i>S. typhosa</i> .....	a" <b>ab'</b>	+
	<i>E. coli</i> .....	a" <b>ab'</b>	++
	<i>Sh. dysenteriae</i> .....	a" <b>ab'</b>	++
Facultative anaerobes	Hemolytic streptococcus.....	0	0
	Viridans streptococcus.....	0	0
	Pneumococcus.....	0	0
Strict anaerobes	<i>Cl. welchii</i> .....	0	0
	<i>Cl. tetani</i> .....	0	0
	<i>Cl. botulinum</i> .....	0	0

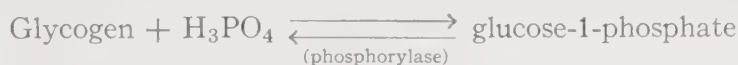
\* Adapted from Fujita and Kodama (1934).

† Adapted from M'Leod and Gordon (1923).

Major components in boldface type.

actions in addition to the decomposition of  $H_2O_2$ . Thus Keilin (1945) has recently demonstrated that catalase will catalyse the oxidation of alcohols by  $H_2O_2$  and can there-

polysaccharidic acids are synthesized by reversal of reactions analogous to the phosphorylase of glycogen. Cori et al. (1942) have shown that the reaction



fore act as a peroxidase. It should be remembered that hydrogen peroxide is produced by autoxidation of many metabolites, such as cysteine, glutathione and flavoproteins.

is reversible. A similar reaction is involved in starch synthesis. It has recently been shown that sucrose is synthesized from glucose-1-phosphate and fructose by *Pseudomonas* and by *Leuconostoc*:



#### POLYSACCHARIDE SYNTHESIS

Many bacterial species synthesize from glucose large amounts of polysaccharide which adhere as an extracellular envelope or capsule to the cell wall. Other specific polysaccharides are located at the surface of or inside the cell. Finally, bacteria may store reserve carbohydrates such as glycogen and starch within the cell. It is probable that most of these polysaccharides and

In some cases, however, polysaccharides can be synthesized in the absence of phosphate (Doudoroff, 1945). Thus the capsular material of *Leuconostoc mesenteroides* is a dextran formed from sucrose. There may be extracted from these organisms soluble enzymes which catalyse the reactions:



and



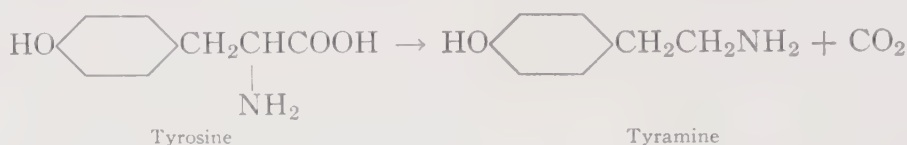


## NITROGEN METABOLISM

Probably all bacteria possess enzymes which hydrolyse proteins. Bacterial proteinases are generally of the papain, trypsin or cathepsin type, active at neutral and slightly alkaline reaction. So far as we are aware, pepsin is not produced by bacteria. Some forms such as clostridia and aerobic spore-forming bacilli may produce very powerful extracellular enzymes of the papain type which break down protein material into more readily assimilable amino acids. The oxidative breakdown of certain amino acids such as glutamic and aspartic

droxyproline can serve as hydrogen acceptors. It is significant that alanine which is oxidized most readily, yields pyruvic acid as its product.

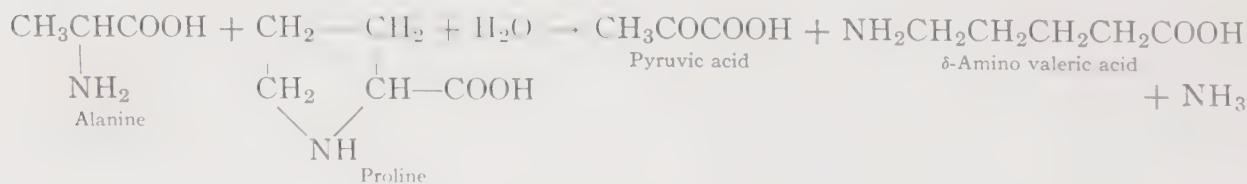
Many bacteria also possess decarboxylases, i.e. enzymes which decarboxylate certain amino acids such as tyrosine, arginine, lysine, ornithine, histidine and glutamic acid. Many of these are adaptive enzymes containing pyridoxal phosphate (or some related derivative of vitamin B<sub>6</sub>) as prosthetic groups. Gale (1946) has shown that there is a specific decarboxylase for each of the six amino acids listed above (see also Table 5). For example:



acids to the corresponding keto-acids, and the transamination reaction of Braunstein and Kritzmann whereby the same amino acids transfer their amino group to pyruvic acid or  $\alpha$ -ketoglutaric acid have already been mentioned in connection with the Krebs cycle. Another reaction which appears to be characteristic of organisms which apparently do not utilize glucose as an energy source, was discovered by Stickland (1935) who demonstrated that *Cl. sporogenes* can catalyse an energy-yielding reaction between two amino acids involving the oxidation of one and reduction of the other. For example:

The decarboxylases are most readily formed and are most active at acid pH (below pH 6) and may be one means used by bacteria to neutralize inhibitory acids formed by fermentation. The reaction could also serve as a source of carbon dioxide. *Cadaverine*,  $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ , is a well-known putrefactive compound, formed by bacterial decomposition of lysine.

There are found in bacteria many other enzymes which attack various amino acids and other nitrogenous compounds. Often the presence or absence of particular enzymes is a useful aid in laboratory diagnosis of bacterial strains or species. Thus *Proteus*



Leucine, valine, aspartic acid and glutamic acid, as well as alanine, can act as hydrogen donors. Only glycine, proline and hy-

*vulgaris* possesses a powerful urease capable of hydrolysing urea to ammonia and carbon dioxide:

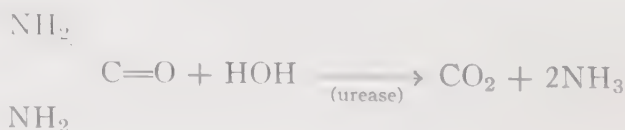


TABLE 5. ADAPTIVE FORMATION OF AMINO ACID DECARBOXYLASES \* IN *Escherichia Coli* †

DECARBOXYLASE	E. COLI STRAIN	ADDITIONS TO MEDIUM							
		NONE	LYSINE	ARGI-NINE	ORNI-THINE	GLUTA-MATE	HISTI-DINE	TYRO-SINE	CASEIN DIGEST
1 (+)-Lysine.....	86	4	210		4				194
1 (+)-Arginine.....	86	0		27					330
1 (+)-Ornithine.....	86	3			225				145
1 (+)-Glutamic acid.....	TY	45				88			100
1 (-)-Histidine.....	86	0					7		18
1 (-)-Tyrosine.....	HE	0						60	63

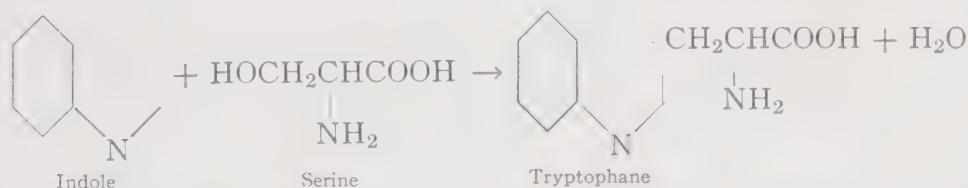
\* Growth medium: Inorganic salt mixture, including  $(\text{NH}_4)_2\text{HPO}_4$  + 2% glucose + additions (1%) as above

† Activities express values of  $\text{Q}_{\text{CO}_2}$  at 30° C. and optimum pH.

(Gale, E. F., 1946, The bacterial amino acid decarboxylases. Advances in Enzymology, 6, 9.)

This reaction is used to distinguish *Proteus* from other morphologically similar Gram-negative bacteria.

Many bacteria, particularly when grown on media rich in tryptophane, produce indole (Hopkins and Cole, 1903). Recently the reverse reaction has been demonstrated (Umbreit et al., 1946)—namely the synthesis of tryptophane from indole and the amino acid serine. The reaction is catalysed by a pyridoxal-phosphate-containing enzyme.



Although indole can be produced from tryptophane by washed cells of *E. coli* (Woods, 1935; Wood et al., 1947), it can also appear as a product of bacterial growth by direct synthesis from ammonia (Fildes, 1938).

Many other nitrogen compounds may be utilized by bacteria. An interesting adaptive enzyme is that described by Dubos and Miller (1937) who showed that cultures of certain soil bacteria grown in the presence of creatinine produce an enzyme decomposing creatinine to urea and other products

in the presence of oxygen. The enzyme is highly specific and forms the basis for the quantitative micro-estimation of creatine and creatinine in blood and urine.

## THE CULTIVATION OF BACTERIA

### PHYSICOCHEMICAL ENVIRONMENT AND INORGANIC REQUIREMENTS

Bacteria differ from the cells of plants and animals in their ability to withstand

relatively large changes in environmental factors such as osmotic pressure, heat and acidity. Naturally, this is particularly true of spores which can often withstand boiling for a considerable length of time, desiccation, freezing, exposure to saturated salt solutions, strong acid and alkali. Vegetative forms can often multiply at very low osmotic pressure—as in tap water containing traces of salts and organic materials, and also in media which would be hypertonic for most plant and animal cells. Hemolytic streptococci, for example, still grow slowly

in the presence of salt concentrations of ionic strength 0.3. Some species, however, are relatively sensitive. Thus gonococci only grow well at osmotic pressures near that of blood plasma (Miller et al., 1932).

Most vegetative forms of pathogenic bacteria die off rapidly at temperatures above 38° C., although some strains survive a considerable length of time at temperatures above 40° C. and even 50° C. Most strains are resistant to cold and can withstand freezing and cooling to very low temperatures. When dried from the frozen state, most bacteria keep indefinitely at ordinary temperatures and can withstand even 100° C. for long periods if perfectly dry.

The pathogens vary widely in their susceptibility to acids and alkalis. In general, growth and multiplication are optimal in the neutral region—i.e., pH  $7 \pm 0.5$ , but growth can occur over a very wide pH range (pH 5-9). Some bacteria, such as those of the acid-fast group, resist treatment with normal mineral acid and alkali. Despite these wide limits of external pH, the interior of the bacterial cell is probably maintained at a relatively constant pH close to neutrality.

The mineral requirements of bacteria resemble those of other cells. Phosphate ions must of course be present for growth. Potassium is also required and has been shown to play an essential part in glycolysis. Calcium ions are required and exert an essential, specific rôle in certain enzyme reactions. The lecithinase produced by *Cl. welchii*, for example, fails to hydrolyse lecithin to phosphorylcholine and diglyceride unless calcium ions are present. Magnesium is essential for growth and is required as a specific catalyst for many enzymes, in particular those containing thiaminepyrophosphate as prosthetic group. Iron is not only necessary for aerobic growth where it is utilized in the formation of iron enzymes such as catalase and cytochromes, but probably also for certain steps in anaerobic glycolysis. Other elements such as copper and

zinc are also essential, but their rôle is not entirely clear at present. Bacteria are capable of concentrating metals such as iron, copper and zinc within the cell, from traces present in the medium. This process obviously requires energy which is presumably derived from glycolysis in most cases.

#### VITAMINS IN BACTERIAL NUTRITION \*

We have already discussed in some detail the essential metabolic processes by which heterotrophic bacteria (including pathogens) obtain energy for their growth and multiplication. Whether this energy is derived anaerobically by fermentation or aerobically by respiration, the same pathways are followed as in cells of higher animals. Most of the enzymes necessary to catalyse the various steps in the energy yielding process contain as prosthetic groups or co-enzymes, various members of the water-soluble vitamins of the B-complex or their derivatives. It is of interest that we find many species of heterotrophic bacteria which have lost their capacity to synthesize these essential metabolites or vitamins and fail to multiply on artificial media which do not contain them. Indeed several of the vitamins now known to be essential for animal or human nutrition were first discovered or isolated as growth factors for micro-organisms. These include nicotinic acid, lack of which causes pellagra in man, pantothenic acid, biotin, folic acid and others.

We have stressed the central position occupied by pyruvic acid in both fermentation and respiration and the great number of reactions which this compound may undergo. Many of these reactions are catalysed by a group of enzymes known as carboxylases. These enzymes are specific proteins, each containing the magnesium salt of thiamine (vitamin B<sub>1</sub>) pyrophosphate or co-carboxylase as their prosthetic group. Thus

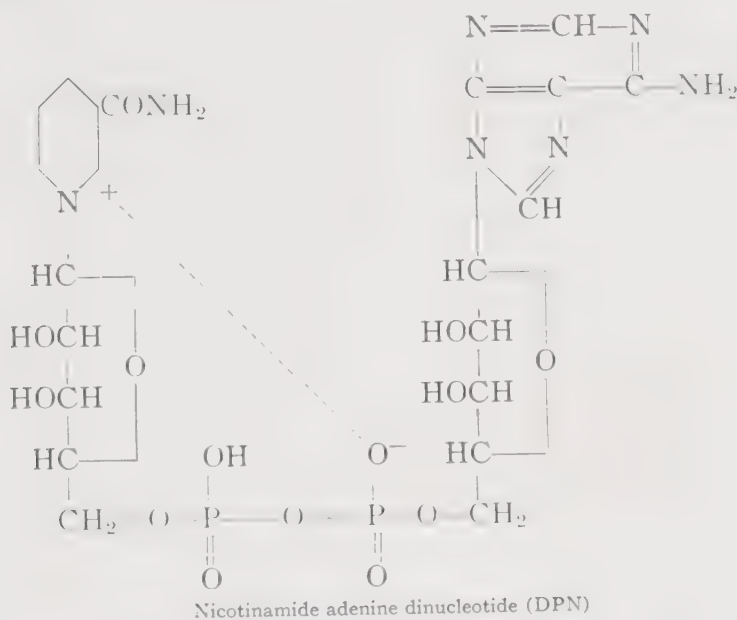
\* For a detailed discussion of bacterial nutrition, the reviews by Knight (1945) and Mueller (1943, 1944) may be consulted.



the decarboxylation of pyruvic acid to acetaldehyde is catalysed by a thiamine-containing enzyme. Thiamine is required for the growth of a great many pathogenic species. Moreover, because thiamine plays an essential rôle in metabolic processes, all strains of bacteria which do not require its presence as a vitamin synthesize it from simpler nutrients. The bacterial synthesis of thiamine is readily demonstrated by the following simple experiment. If we inoculate the diphtheria bacillus in a medium lacking thiamine but otherwise sufficient, luxuriant growth occurs. The same medium, however, fails to support growth of most strains of staphylococci, unless thiamine is added. If the diphtheria bacillus is allowed

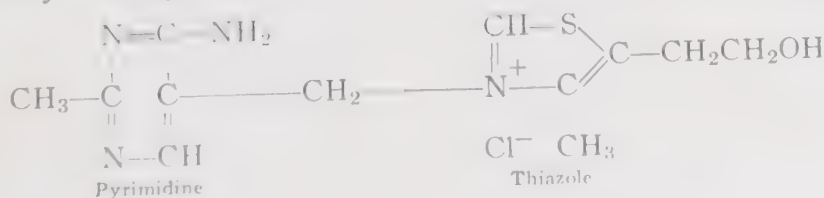
While some organisms require the intact thiamine molecule for their growth, it has been found that many species, including *Staphylococcus aureus*, can multiply equally well if both pyrimidine and thiazole moieties are supplied separately. Still other strains have been found which can synthesize the complete thiamine molecule provided the pyrimidine component is present in the medium; still others from the thiazole moiety alone.

Several of the steps in the fermentation and respiration schemes involve reversible oxidations catalysed by dehydrogenases which contain phosphopyridine nucleotides (DPN and TPN) as dissociable prosthetic groupings:



to grow for 48 hours in the thiamine-deficient medium and the organisms are then removed by filtration, the sterile filtrate supports excellent growth of staphylococcus thus demonstrating thiamine synthesis by *C. diphtheriae*.

The thiamine molecule is composed of a pyrimidine and a thiazole component linked together by a methylene bridge:

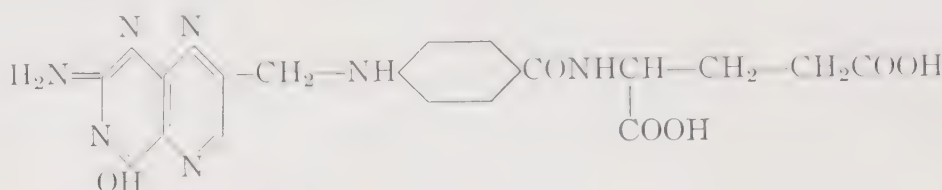


Oxidations of lactic to pyruvic acid, 1,3 phosphoglyceraldehyde to 1,3 phosphoglyceric acid or of malic to oxaloacetic acid are all reactions requiring DPN containing enzymes. A great many bacteria fail to grow unless certain of the specific chemical groupings contained in the DPN molecule are incorporated into the medium. Thus, many strains of staphylococci and of the

diphtheria bacillus require nicotinic acid for their growth. Certain strains of *Pasteurella*, however, cannot synthesize phosphopyridine nucleotides from nicotinic acid but require nicotinamide. The influenza bacillus is even more fastidious and fails to grow unless nicotinamide nucleoside or the complete coenzyme is present in the medium. Still other species require adenine or a related purine as an essential growth factor. From the nutritive requirements of various bacterial species with regard to DPN it is

7 are presented some of the enzyme systems of which the prosthetic groups contain vitamins, or specific portions of them, essential for growth of many strains of pathogenic bacteria.

In addition to vitamins whose function in certain types of metabolic reactions is known, micro-organisms require various other essential growth factors for which no definite role has as yet been discovered. One of these is folic acid or pteroylglutamic acid:



possible to indicate certain of the steps in the bio-synthesis of the coenzyme as illustrated by Table 6 (Knight, 1945).

When DPN is reduced by a substrate such as lactic acid, the reduced DPN may be reoxidized by flavoprotein, an enzyme which contains riboflavin-adenine dinucleotide as its prosthetic group. Riboflavin (vitamin B<sub>2</sub>) is an essential growth factor for many bacterial species including hemolytic streptococci and pneumococci and once again organisms which do not require riboflavin are able to synthesize it. In Table

It will be noted that this vitamin contains p-aminobenzoic acid joined to glutamic acid by a peptide linkage on the one hand and to the pterin grouping on the other. p-aminobenzoic acid was first isolated as an inhibitor of the action of the sulfonamide drugs and later was discovered as a growth factor for certain bacteria (*Acetobacter suboxydans* and *Cl. acetobutylicum*) before its identification as a portion of the folic acid molecule was realized. Certain micro-organisms, however, such as *Lactobacillus casei* apparently require the intact folic acid

TABLE 6. NUTRIENT REQUIREMENTS OF BACTERIA AS RELATED TO PHOSPHOPYRIDINE NUCLEOTIDE SYNTHESIS

$  \begin{array}{c}  \text{NH}_3 \xrightarrow{1a} \\  \text{Amino acids} \xrightarrow{1b}  \end{array}  \rightarrow \text{nicotinic acid} \xrightarrow{2} \text{nicotinamide} \xrightarrow{3} \text{nicotinamide nucleoside} \xrightarrow{4} \text{DPN, TPN}  $		
ORGANISM	MINIMAL NUTRIENT REQUIREMENT WITH REGARD TO DPN STRUCTURE	STAGES OF SYNTHESIS WHICH CAN BE CARRIED OUT
<i>E. coli</i> (certain strains) . . . . .	NH <sub>3</sub>	1a, 2, 3, 4
<i>S. typhosa</i> . . . . .	Amino acids	1b, 2, 3, 4
<i>Proteus vulgaris</i> . . . . .	NH <sub>3</sub> + nicotinic acid	2, 3, 4
<i>Staph. aureus</i> . . . . .	Amino acids + nicotinic acid	2, 3, 4
<i>C. diphtheriae</i> . . . . .	Amino acids + nicotinic acid	2, 3, 4
<i>Pasteurella</i> sp. . . . .	Amino acids + nicotinamide	3, 4
<i>H. influenzae</i> . . . . .	Nicotinamide nucleoside	4
<i>H. parainfluenzae</i> . . . . .	Nicotinamide nucleoside	4

TABLE 7. VITAMINS WHICH BEHAVE AS PROSTHETIC GROUPS OF ENZYME SYSTEMS AND AS GROWTH FACTORS FOR BACTERIA

ENZYME OR TYPE OF REACTION CATALYSED BY AN ENZYME	PROSTHETIC GROUP OR COENZYME	VITAMIN OR PORTION OF VITAMIN REQUIRED
1. Carboxylases	Magnesium salt of thiamine pyrophosphate	Thiamine (vitamin B <sub>1</sub> ) Thiazole Pyrimidine
2. Various dehydrogenases	Diphosphopyridine (DPN) Triphosphopyridine nucleotides (TPN)	Nicotinic acid Nicotinamide Nicotinamide riboside Adenine
3. Flavoproteins Amino acid oxidation Purine oxidation DPNH <sub>2</sub> oxidation	Riboflavin adenine dinucleotide	Riboflavin (vitamin B <sub>2</sub> ) Adenine
4. Cytochromes a, b, c Catalase	Hemin	Hemin or protoporphyrin + Fe
5. Transaminases Amino acid decarboxylases Tryptophane synthesis and breakdown	Pyridoxal phosphate	Pyridoxine (vitamin B <sub>6</sub> ) Pyridoxamine Pyridoxal
6. Acetylation	Pantothenic acid derivative	Pantothenic acid $\beta$ -Alanine $\alpha$ -Hydroxy- $\beta$ -dimethyl- $\gamma$ -butyrolactone
7. CO <sub>2</sub> fixation	Biotin derivative(?)	Biotin Pimelic acid

molecule. Other growth factors for bacteria whose role in metabolism is still not clear include vitamin K, choline, inositol, streptogenin (a glutamic acid-containing peptide required for growth of certain strains of hemolytic streptococci of Group A) and probably other factors. With the exception of vitamin K, none of the fat-soluble vitamins (A, D, E) have been found in bacteria. Vitamin C (ascorbic acid) has been reported essential for growth of certain spirochaetes.

In general the vitamin requirements of bacteria closely resemble those of higher animals and where a vitamin is not required for growth of a given strain, it can usually be shown that the organism in question is capable of synthesizing it from simpler ma-

terials. An exception to this rule is, of course, hemin which is only found in aerobic organisms, where it is the prosthetic group of catalase and of the cytochromes.

It is worth mentioning at this point that vitamin synthesis by bacteria of the intestinal tract probably constitutes a major source of certain vitamins in human and animal nutrition. Thus, *E. coli* grown on a synthetic medium produces 1,000 units vitamin K per gram-dry bacteria, and it is probable that most of the vitamin K in human nutrition is derived from bacterial synthesis rather than from food. Administration of large amounts of sulfonamide drugs prior to gastro-intestinal surgery may occasionally reduce the bacterial population of the intestine to such an extent as to make



operations dangerous unless vitamin K is administered. A large portion of our pantothenic acid intake is also probably derived from bacterial synthesis in the intestine. In cattle this is unquestionably the principle source of this vitamin which is synthesized in large amount by bacteria of the rumen.

#### AMINO ACID REQUIREMENTS \*

The capacity of heterotrophic bacteria to synthesize amino acids varies all the way from organisms such as *E. coli*, most strains of which require only ammonia for their growth, to those highly fastidious forms exemplified by hemolytic streptococci of group A and *Leuconostoc mesenteroides* which must be supplied with almost the entire gamut of amino acids before growth can take place. In general, the amino-acid requirements of the Gram-negative intestinal parasites are not exacting. Many strains of *S. typhosa*, when freshly isolated, require only the amino acids tryptophane and cystine. It is not difficult, however, to isolate from such cultures, mutants which can synthesize these amino acids from ammonia. The amino acids most frequently required by the more fastidious forms, especially the Gram-positive bacteria, appear to be those containing specific groupings such as tryptophane, cystine, methionine, tyrosine, phenylalanine and histidine. Glutamic acid, aspartic acid and alanine are rarely required by bacteria, although strains have been found which are unable to synthesize them.† Of the 21 common amino acids, only hydroxyproline, norleucine and norvaline have not been found essential for growth of any bacterial strain. Amino acid requirements are generally highly specific, and it is usually not pos-

sible to replace one amino acid with another. For example, an organism requiring tyrosine for its growth will not often accept the closely related amino acid, phenylalanine, as a substitute.

The specific requirements of certain bacteria make them exceptionally suitable for microbiologic assay of amino acids in proteins. *Leuconostoc mesenteroides*, for example, manifests a specific need for no fewer than 17 different amino acids. By cultivation of this organism on a medium containing 16 amino acids in excess, together with the necessary vitamins, carbohydrate and salts, the remaining amino acid in an unknown mixture can be accurately titrated by measurement of its growth promoting activity. Microbiologic assay thus provides one of the most rapid and accurate methods at present available for estimation of the amino acid composition of proteins. Analogous methods for microbiologic assay of most of the vitamins of the B group have also been developed.

It is difficult to generalize regarding the specific amino acid requirements of different species of bacteria since even strains within a given species vary greatly in this respect. Moreover, mutations which give rise to variants either lacking or requiring a given amino acid occur with some frequency in laboratory cultures, a fact which constitutes a serious source of error in the microbiologic assay method. Stock cultures for assay work must be constantly checked to eliminate the possible selection of less exacting mutants with respect to amino acids and vitamins.

The requirement for glutamic and aspartic acids discussed above is also interesting in that some species require these amino acids in the form of their amides. Glutamine is required for growth by many strains of hemolytic streptococci of group A and asparagine by some strains of pneumococci.

\* An extensive review on amino acid requirements has been written by Snell, 1945.

† It is obvious that such strains must lack enzymes concerned in transamination or reductive amination since they would otherwise be able to synthesize these amino acids from pyruvic acid, CO<sub>2</sub> and ammonia.

## FACTORS AFFECTING INITIATION OF GROWTH

The energy, amino acid and vitamin requirements of bacterial growth have already been considered. In addition to these, a number of other factors have been found to influence the initiation of growth, particularly from minute inocula containing only a few living cells.

It is obvious that the presence of a toxic substance can retard or completely prevent the development of the culture, but it is worth emphasizing that many essential substances can become toxic under certain circumstances. Thus, a solution containing sodium chloride as the only electrolyte is bactericidal for all bacterial species and ions must be present in properly balanced concentrations for normal physiologic performance. Similarly, an essential amino acid, methionine for example, can become inhibitory if present in too high concentration, although its inhibitory effect can be neutralized merely by adding to the medium other amino acids such as alanine and lysine (Mueller, 1944). Long-chain fatty acids, especially the unsaturated ones, are extremely toxic to many bacterial species; pneumococci, streptococci, meningococci, gonococci, diphtheria bacilli, tubercle bacilli, etc., but this toxicity can be decreased or overcome by adding to the medium adequate amounts of starch or of certain proteins, serum albumin, in particular. It appears that these substances can form with fatty acids—as well as with other toxic products—complexes which are innocuous to the cell. Thus, there are probably many cases where the beneficial effect on growth of complex organic substances results not from the addition of essential nutrients, but rather from the neutralization of potentially toxic effects (Davis and Dubos, 1947).

It is frequently observed that initiation of growth is facilitated when reducing conditions prevail in the medium. This is true

for strictly anaerobic species as well as for pneumococci and streptococci and even for certain organisms which, like staphylococci, can utilize oxygen in their metabolism. The oxidation reduction potential of the medium can be conveniently lowered by adding to the latter reducing substances such as reduced iron, cysteine, thioglycolic acid, ascorbic acid, etc. Oxidation of the SH groups, as well as of other autoxydizable constituents of the medium, is naturally prevented by eliminating oxygen from the system by either physical, chemical or biological methods.

There are a number of growth promoting factors which are not absolutely essential but which accelerate growth because the organism can synthesize them only at a slow rate. Carbon dioxide is of special interest in this respect, since it is essential for the growth of many species although it is produced by all of them. If the environment contains enough  $\text{CO}_2$  to allow initiation of cellular multiplication, growth will continue provided the metabolic  $\text{CO}_2$  is not removed from the system. A similar situation obtains with certain unsaturated fatty acids which are synthesized at such a slow rate that they may constitute limiting factors of growth rates and that their absence can, in extreme cases, completely prevent initiation of growth of small inocula. The diphtheria bacillus for example, will grow in the absence of oleic acid if the inoculum is heavy. When they are grown on agar surfaces from small inocula, on the other hand, growth is poor and greatly delayed unless small amounts of oleic acid are added to the medium (Mueller, 1944). Long-chain fatty acids have a similar effect on the growth of tubercle bacilli and of other organisms but, as already mentioned, higher concentrations of fatty acids are toxic for these same organisms.

Minimal growth requirements are usually the more exacting the smaller the inoculum used in the test. There are many independ-



ent reasons for this finding. Large inocula introduce into the new medium some of the substances required for growth. Carbon dioxide and reducing substances produced during growth, metabolites which are synthesized with difficulty, etc., may be supplied in amounts adequate for growth if the inoculum contains a sufficient number of cells. Toxic substances, if present in large enough concentration, may be completely bound by some of the cells of the inoculum which thereby protect the surviving population. Finally, it must be realized that the use of large inocula facilitates adaptation of the culture to a deficient medium by virtue of the heterogeneity of the bacterial population. As we shall see, bacterial cultures often give rise to variant forms, less exacting in growth requirements than the majority of the cells in the culture. Thus typhoid bacilli recently isolated from pathologic material cannot grow in the absence of tryptophane; most typhoid cultures, however, produce variants capable of synthesizing this amino acid. Inoculation of a medium deficient in tryptophane, with an inoculum large enough to contain the variant form, will give rise to growth where a smaller inoculum would fail to multiply. A similar situation has been described in the case of *Johne's bacillus*, which requires extracts of acid fast bacilli for growth on primary isolation but which will grow slowly without these extracts when large inocula are used in subsequent transplants.

#### FACTORS AFFECTING TOTAL YIELD OF GROWTH AND VIABILITY OF CULTURE

Given a medium in which initiation of growth is possible, the yield of growth will naturally depend upon an adequate supply of energy sources, structural constituents and accessory growth factors. These have already been considered and need not be discussed further. It must be emphasized, however, that the final density of the culture does not depend only upon the con-

centration of nutrients in the medium. Thus, oxygen deficiency may prevent the complete oxidation by aerobic organisms of the potential sources of energy. The rate of respiration in a growing aerobic culture is so rapid that oxygen diffusion through the medium is usually too slow to prevent the conditions from becoming anaerobic even when the culture apparently has free access to air. It is possible to increase the oxygen supply by violent shaking or forced aeration of the culture during incubation; yields of bacteria can thus be increased manyfold over those obtained in still cultures.

Incomplete oxidation of carbohydrates, due for example to inadequate supply of oxygen, results in the accumulation of organic acids in the culture medium. Organic acids accumulate also, even with adequate aeration, in cultures of bacterial species (*pneumococci*, *streptococci*, anaerobes, etc.) which are deficient in respiratory catalysts (cytochrome system). Even when the acidity is neutralized with proper addition of alkali, accumulation of organic acids eventually causes inhibition of growth as soon as the concentration of organic salts becomes too high. Toxic metabolic products other than organic acids are often produced during growth; for example hydrogen peroxide can accumulate to toxic levels in cultures of organisms deficient in catalase (*pneumococci*, anaerobes) grown in the presence of air.

#### CRITERIA FOR THE SELECTION OF CULTURE MEDIA

It is difficult to formulate a single medium ideal for all purposes, even in the case of a single bacterial species. For diagnostic purposes, for example, the most important criterion is that the medium should allow detectable growth of as small an inoculum as possible (preferably a single cell) within the shortest possible period of incubation. Where, on the other hand, large yields of



growth are desired, primary consideration should be given to those environmental factors which permit an extended period of logarithmic growth and which allow the attainment of high bacterial densities. Maintenance of viability for the preservation of cultures demands that emphasis be placed on still other properties of the medium. The great variability of bacterial cultures introduces further requirements which, in most cases, have not yet been satisfied. There is great need for a better understanding of the factors which favor the growth and determine the selection of the multiple possible variants of any given bacterial species. Finally, it should be mentioned that special problems bring forth added criteria to be fulfilled. Thus, the chemical isolation of toxins or other antigens and metabolic products and the study of nutritional requirements and of metabolic activity of bacteria are rendered much easier when the culture is grown in a medium of minimal complexity and of known composition. In each case, therefore, the goal to be achieved and the peculiarities of the organism studied have to be taken into consideration for the selection of the most satisfactory culture medium.

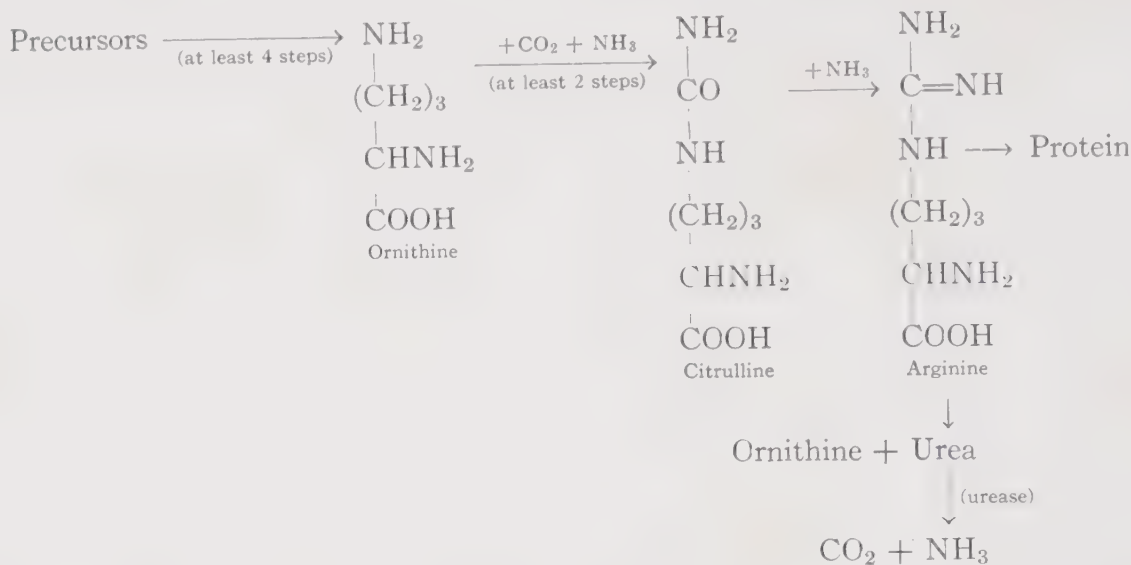
#### BIOSYNTHESIS

In a preceding section we indicated how various steps in the biosynthesis of certain coenzymes could be deduced from a study of specific requirements of different strains of bacteria for certain vitamins. Thus, Table 6 indicates at least 4 steps from ammonia or amino acids through nicotinic acid to its amide, through nicotinamide-riboside and finally to DPN or TPN. Recently it has been found possible to induce mutations in bacteria and molds by physical methods, particularly ultraviolet light, X-radiation and by poisons such as the nitrogen mustards. These agents alter hereditary factors (or genes) controlling synthesis of enzymes concerned in specific reactions and cause the

production of mutants which differ from the parent culture (wild type) by their inability to carry out a particular biosynthetic reaction. Most strains of *E. coli*, for example, grow well on media which contain only glucose and ammonia, in addition to inorganic constituents. By successive treatment of such cultures with X-rays or the nitrogen mustards, mutants can be isolated which show increasingly complex requirements with regard to vitamins and amino acids. Most of the work in this field (Beadle, 1945; Tatum, 1947) has been carried out, not with bacteria, but with the mold *Neurospora crassus* which lends itself to such studies because the parent or "wild" type has exceedingly simple nutritive requirements and because changes in individual genes are readily followed by the geneticist. This mold is bisexual, and the crossing of two parent strains results in the formation of four primary meiotic nuclei which give rise to eight ascospores arranged in a row. Each ascospore can be dissected out and inoculated into a suitable medium for study of its growth requirements. It has thus been possible to show that specific reactions involved in the synthesis of growth factors and amino acids are gene-controlled.

The biologic formation of arginine illustrates the utilization of this method to identify the steps in biosynthesis. The parent strain of *Neurospora crassus* does not require this amino acid for its growth and is capable of synthesizing it from simpler compounds. By X-irradiation, Srb and Horowitz (1944) have isolated distinct mutants requiring arginine as an essential amino acid, each differing from the wild type by a single gene. One of these mutants will grow only when the medium contains arginine itself. Two more mutants grow satisfactorily when arginine is replaced by citrulline but not by ornithine. The remaining four mutants apparently regulate steps in the synthesis of ornithine which as yet have not been identified. It thus appears that the mold, *Neurospora crassus*, synthe-

sizes arginine through ornithine and citrulline as postulated previously for mammalian liver by Krebs:



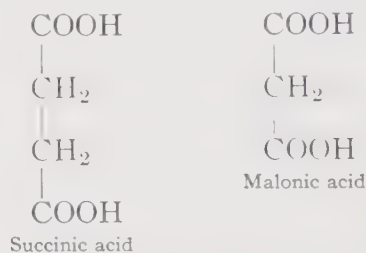
Work of this kind is now in progress, using bacteria as well as *Neurospora*, and is being applied to disclosing the steps in synthesis of many other amino acids and vitamins. It should be stressed that this is only one of the methods available for gaining insight into the pathways of biosynthesis. The use of labelled or marked atoms or groupings by means of radioactive tracers or isotopic carbon, hydrogen and nitrogen has provided powerful tools for investigation of both anabolic and catabolic pathways.

#### COMPETITIVE INHIBITION \*

Since most of the water soluble vitamins are required for growth by virtue of groupings essential for important enzyme reactions, it is obvious that any compound which can compete with a vitamin or coenzyme for its position on an enzyme molecule should have a deleterious effect on bacterial growth. In fact, it has long been known that compounds which are structurally or stereochemically related to one another do compete in this manner. For example, the oxidation of succinic acid to fu-

maric acid is catalysed by the enzyme succinate dehydrogenase. Succinate oxidation may be completely inhibited by relatively small

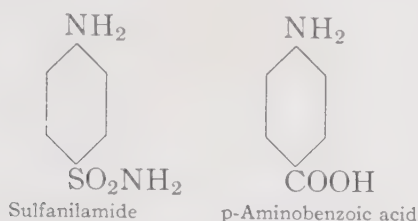
amounts of the closely related dicarboxylic acid salt, malonate:



Presumably both malonate and succinate compete for the same enzyme, succinate dehydrogenase. The enzyme, however, is unable to oxidize malonate which can therefore act as an inhibitor by combining with the enzyme and preventing it from reacting with succinate. The inhibitory action of malonate can be reversed by raising the succinate concentration. It was suggested by Fildes (1940) that the action of the sulfonamide drugs might be due to their structural similarity to an essential vitamin or metabolite. Drug and essential metabolite might compete for a position on the same enzyme or protein carrier (apo-enzyme). Fildes' prediction was verified soon afterward when Woods (1940) isolated p-aminobenzoic acid from yeast and showed that the bacteriostatic action of the sulfonamide drugs

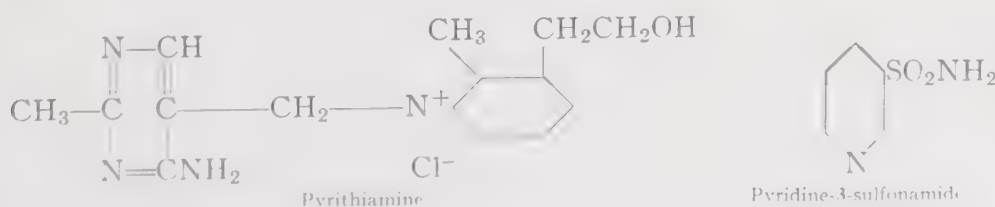
\* See review by Woolley, 1946.

could be reversed by small amounts of this compound. Later p-aminobenzoic acid was found essential for the growth of certain bacteria and still later was found to be an integral portion of the folic acid molecule (pteroylglutamic acid).



Both sulfanilamide and p-aminobenzoic acid presumably compete for the same enzyme protein, possibly concerned in the synthesis of pteroylglutamic acid. The relative effectiveness of the drug in inhibiting bacterial growth is dependent on the relative dissociation constants of the drug-enzyme and p-aminobenzoic acid-enzyme complexes. The competition is analogous to that of carbon monoxide and oxygen for hemoglobin where the amount of carbon monoxide combined depends on the relative partial pressures of oxygen and carbon monoxide and the dissociation constants of oxyhemoglobin and carboxyhemoglobin. Likewise, some "antihistamine" drugs may act by virtue of their structural likeness to histamine (page 149).

The work of Fildes and Woods has stimulated the synthesis of many compounds structurally related to vitamins and amino acids. However, very few of these have proved effective in vivo; two further examples of such artificial inhibitory structural analogs are *pyrithiamine* related to thiamine and *pyridine-3-sulfonamide* related to nicotinic acid both of which inhibit bacterial growth:



## GROWTH CYCLES AND BACTERIAL VARIABILITY

### GROWTH OF BACTERIAL CULTURES

The development of a bacterial culture inoculated into an adequate nutrient medium can be described in terms of a number of successive phases characterized by the rate of cellular multiplication and by the morphologic and biologic properties of the individual cells. The following phases of the growth cycle are usually recognized: the lag phase, during which no appreciable cellular multiplication takes place; the logarithmic phase, during which the number of living cells increases logarithmically; the stationary phase, during which it remains approximately constant; the phase of decline, during which the number of living cells decreases more or less rapidly.

There is no evidence that the growth cycle and its constituent phases correspond to intrinsic biologic characteristics of the bacterial species under consideration. The growth curve is only the resultant of a multiplicity of factors—many of them unrelated—which have influenced the past history of the culture, and which condition the fitness of the environment for its growth. The curve representing the growth cycle is only a convenient mathematical summary of the composite effect of these factors on the number of living cells present in the culture at any given time; its shape can be changed at will by altering any one of these factors. The effects of the composition of the medium, and of variation in the enzymatic equipment of the culture, on the different phases of the growth curve, are described in the following pages. A modern quantitative treatment



of the problem of growth phases will be found in Monod (1942).

#### PHYSIOLOGIC CHARACTERISTICS CORRELATED WITH GROWTH PHASES

Changes in the rate of bacterial multiplication—corresponding to the different phases of the growth cycle—are often correlated with alterations of some of the morphologic and biologic properties of the cells. Thus, there often occurs a marked elongation and increase in total volume of the individual cells during the lag phase, i.e., at a time when there is no increase in cell numbers; in other words, growth as expressed by synthesis of new protoplasm can occur unaccompanied by cellular division. During the lag period also the growing but not multiplying cells exhibit high metabolic activity. This can be measured in terms of  $O_2$  uptake and  $CO_2$  production, of increased ability of the cells to adapt themselves to the utilization of new substrates (production of adaptive enzymes), and finally of increased susceptibility to toxic substances, to heat, and to other inimical conditions in the environment.

The individual cells are small in size during the logarithmic phase when the process of cell division takes place as rapidly as the synthesis of new protoplasm. When transferred to an adequate medium they can generally initiate growth without any lag period.

The stationary phase corresponds to the time during which the rate of death is approximately equal to that of production of new cells; from then on, the culture reaches progressively the phase of declining numbers. Exhaustion of nutrients and accumulation of toxic products exert obscure and complex effects on the properties and fate of the individual members of the population. The cells of the stationary and declining phases are usually of small dimensions, poorly able to adapt themselves to new substrates and slow to initiate growth when

transferred to new media. Interestingly enough, they often exhibit higher resistance than the young active forms to inimical environmental conditions; they appear to behave in some respects as resting stages of the organism.

These alterations in the morphologic and biologic characteristics which are correlated with the different phases of the growth cycle have been summarized by the statement that bacteria can exist in different physiologic conditions corresponding to embryonic state, physiologic youth, and state of senescence. Granted that these expressions are justified only as analogies and reflect a multiplicity of complex phenomena, they serve to emphasize that the physiologic activity of a given bacterial cell can undergo profound modification. In addition to its interest for the general problems of cellular biochemistry, this fact has significance for the understanding of pathogenic behavior. For example, it has been observed that smaller numbers of pneumococci and streptococci are required to establish an experimental infection in mice when the bacteria are in the logarithmic phase of growth than when older cells are used for establishing the infection. It is very likely that similar factors play a part in the communicability of infectious diseases under natural conditions. The decreased susceptibility of bacteria to toxic agents during the later phases of growth is equally a factor of importance for the problems of disinfection and chemotherapy. It has been repeatedly shown, for example, that penicillin is only bacteriostatic for resting organisms, whereas it exerts a bactericidal effect on multiplying cells.

#### ADAPTIVE PRODUCTION OF ENZYMES

In addition to the more-or-less cyclic variations which they undergo in the course of their growth cycle, bacteria can manifest reversible alterations of their appearance and properties induced or favored by

modifications in the composition of the medium. Among the reversible alterations of the cell, some result in a modification of enzymatic activity which appears to correspond to an adaptive response to the presence of certain nutrients in the medium. It was shown in Table 5, for instance, that the decarboxylase activity of *E. coli* can be increased selectively with reference to a given amino acid. Similar phenomena of adaptive production of enzymes have been recognized in the case of many microbial species and affect a great variety of hydrolytic and oxidative enzymes. It is convenient to designate as adaptive those enzymes the production of which is stimulated when the organism utilizes the corresponding substrate, and as constitutive those which appear to be more permanent attributes of the cell and are formed independently of the composition of the medium (Karstrom, 1937). It must be pointed out, however, that there is increasing evidence that the concentration in the cell of the so-called constitutive enzymes can also undergo some variations when the cultural conditions bring about changes in metabolic behavior, and that the difference between the two groups of enzymes is of a quantitative rather than qualitative nature.

The possible mechanisms of enzyme adaptation have been discussed critically in several recent reviews (Dubos, 1945; Gale, 1943; Lwoff, 1946) and cannot be considered here. It appears worthwhile, however, to mention the phenomenon of diauxis, recently discovered by Monod (1942), which reveals that adaptation of all specific adaptive enzymes attacking carbohydrates in bacteria is inhibited by other carbohydrates, which are attacked by constitutive enzymes. When bacteria are grown in a synthetic medium with a mixture of two carbohydrates, one of which is attacked by an adaptive, the other by a constitutive enzyme, growth is characterized by two complete growth cycles, separated by a lag period. Regardless of which substrate the bacteria may have been adapted to previously, the first growth cycle always corresponds to assimilation of the constitutive substrate, the second to the adaptive substrate.

When  $L^+$  bacteria, previously grown on lactose, are inoculated into a lactose-glucose medium, the typical "diauxic" growth curve is obtained, whereas growth of  $L^-$  bacteria is of course limited to the first cycle, corresponding to the assimilation of glucose (Chart 1). It is seen from the growth curve that the suppression of lactose adaptation is quite complete. A definite lysis, characteristic of the absence of an assimilated carbon source, occurs during the lag period separating the two growth cycles.

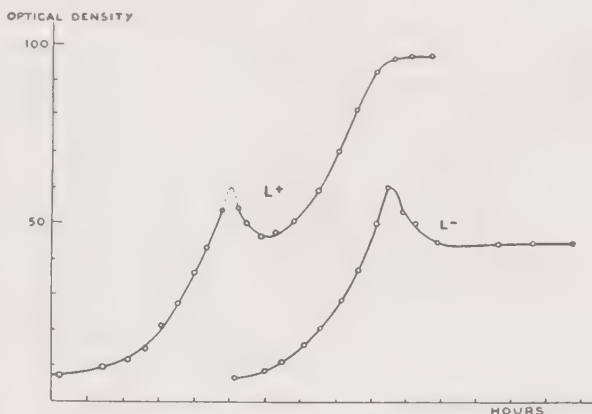


CHART 1. Growth of  $L^-$  and  $L^+$  *Escherichia coli mutabile* on a mixture of glucose and lactose (1 per 10,000 each).  $L^-$  bacteria utilize only glucose.  $L^+$  bacteria show the typical "diauxic" curve: (1) utilization of glucose, (2) lysis due to inanition, (3) adaptation and utilization of lactose. (J. Monod and A. Audureau, 1946.)

These results show that gain by  $L^-$  bacteria of the ability to attack lactose involves the functioning of two mechanisms, one of which is spontaneous and irreversible, the other substrate-induced and entirely reversible.  $L^-$  bacteria do not seem to differ from  $L^+$  bacteria by the possession of a "new" enzyme, but rather by the faculty of adaptation of a precursor. The results further show that adaptation of this precursor may be inhibited or suppressed by other carbohydrates, such as glucose, the attack of which however depends on *another specific enzyme*. This appears in particular from the fact that both  $L^-$  and  $L^+$  bacteria attack glucose with practically equal intensity (Lwoff, 1946).

It is likely that the enzymatic equipment of pathogenic bacteria growing in vivo in the presence of animal tissues differs from



that of the same organisms growing in artificial media in vitro, and it is possible that this difference is of significance in explaining some of the obscure phenomena of infection. Thus, hyaluronidase (spreading factor) is produced adaptively by several pathogenic organisms as a response to the presence of hyaluronic acid (a tissue constituent) in the medium, and this enzyme is known to play a part in certain pathologic processes.

#### HEREDITARY VARIATIONS

Whereas adaptive enzyme production is a specific and reversible response to the chemical composition of the medium, and ceases as soon as the cell is returned to a medium which does not contain the homologous substrate, there are other modifications of the metabolic equipment of the cell which exhibit a more permanent and hereditary behavior. The first example to be investigated was that of *E. coli mutabile*, a species in which the parent culture does not ferment lactose but which gives rise by variation to a population endowed with the enzyme lactase, when it is grown in the presence of this disaccharide. In this case the culture remains henceforth lactose positive even when transferred into media free of lactose. It has been shown that lactose-negative strains can give rise spontaneously to lactose-positive mutants even in the absence of lactose. Variability has been recognized in all microbial species, and affects practically all of their known characteristics; the morphology and chemical structure of the cell, and hence its immunologic specificity; its ability to hydrolyze, oxidize and utilize new substrates, to synthesize growth factors and metabolic products, thus resulting in modification of nutritional requirements and of production of biologically active substances; its susceptibility to various types of inimical factors; its ability to invade foreign hosts and to cause disease, etc. The multiple manifestations

of bacterial variability and the mechanism of their hereditary transmission constitute therefore one of the most important aspects of medical bacteriology. We shall briefly review a few of the modalities of the phenomenon and the terminology under which it has been recognized and described.

#### ADAPTATION AND TRAINING

It is often possible to render a given bacterial culture more resistant to one or another substance or to various inimical environmental factors such as high osmotic pressure, by transferring the culture into media containing progressively increasing concentrations of the toxic agent under consideration. Conversely, certain bacterial species which exhibit complex nutritional requirements when first isolated from pathologic material can be rendered less exacting by transferring them into media containing progressively lower concentrations, and finally none, of some growth factor which appeared essential at the beginning. We have already mentioned (p. 44) the case of typhoid bacilli and of Johne's bacilli which become less exacting in their growth requirements after repeated transfers in laboratory media. Bacteria can be modified not only with reference to their ability to grow in new media, but also in their behavior toward animal hosts, and it is often possible to increase the virulence of certain bacterial pathogens for an animal host by repeated passage of the culture through that host.

All these variations appear to correspond to a progressive adaptation of the microorganism to a new environment. One can often control at will by experimental procedures the direction and extent of the adaptation, and, for these reasons, the phenomena are often described under the name of training. According to this terminology, one can train the bacterium to grow in a new medium, or to increase its virulence



for a given host. The mechanism of the phenomenon will be considered on page 52.

### BACTERIAL DISSOCIATION

Pure cultures of bacteria frequently produce multiple types of growths which can be readily differentiated by the morphologic characters of the colonies growing on solid media. This phenomenon, known as bacterial dissociation, has been observed with cultures issued from single cells, and occurs among saprophytic as well as parasitic species; it corresponds therefore to some fundamental property of the cell. There has grown up a complex terminology for the description of colonies, particularly with reference to the texture of their surface: mucoid, smooth, rough, rugose, matt, glossy, etc. (often abbreviated as M, S, R, R, G, etc.). Other modifications of the colonies are determined by changes in the rate of growth which can result, for example, in the production of small-colony variants often designated as D (dwarf). Still other types of variation affect pigmentation, both qualitatively and quantitatively. Many of these manifestations of colonial morphology are correlated with the immunologic and pathogenic properties of the bacteria concerned, and medical bacteriologists have, therefore, devoted much effort to the description of the different dissociation phases of pathogenic species. Unfortunately, the terminology used for the description of colony variants is based on such gross morphologic criteria and has grown in such a haphazard manner that any attempt to transfer to another microbial species the knowledge acquired with a specific organism is often misleading. For example, pneumococci, streptococci, typhoid bacilli, and many other species can all produce mucoid colony variants; it is probable, however, that the mucoid character does not have the same significance in the fundamental biology of the cell in these different cases,

and it is certain that it does not bear the same relation to pathogenicity.

The confusion in terminology has rendered even more difficult the analysis of the fundamental nature of bacterial dissociation. According to one theory, the different dissociation phases of a given culture correspond to more or less stable stages in an orderly development cycle of the culture. It is more commonly believed, however, that the dissociation phases are more analogous to discontinuous variations of independent structural characters of the cell which depend on the spontaneous appearance of variants (mutants) and their subsequent establishment under the control of the inherent and environmental factors which govern population dynamics. Extensive and critical reviews of the phenomenon of bacterial dissociation have been given by Hadley (1931, 1939) and Braun (1947).

### VARIATION IN ANTIGENIC STRUCTURE

Many of the changes in colonial morphology which are described under the name of bacterial dissociation correspond to hereditary changes in surface components of the cell.

Thus the mucoid character is often associated with the presence of a capsule consisting of high molecular weight hydrophilic substance (usually polysaccharide in nature). Loss of the mucoid character results from failure by the cell to form, or at least to accumulate, this capsular material. In general the R (rough) variants are also derived from the S (smooth) forms by the loss of ability to produce a certain surface component. The chemical nature of this substance characteristic of the smooth phase varies with each bacterial group and type and is often used by immunologists to define the serologic specificity of the culture; passage from the S to the R phase is accompanied therefore by loss of the immunologic specificity due to the surface antigen.



As a result of their motility, some flagellated species have a tendency to "spread" over the surface of solid agar media to give a thin film of growth. This spreading growth was first described by the German workers with the word "Hauch." The corresponding bacterial forms are often described as H forms, and the flagellar material is designated as H substance. Loss of flagellation is correlated with loss of the H substance and failure to give the spreading growth. In contrast with the spreading forms, the nonspreading variants were described by the expression Ohne Hauch (abbreviated as O). With increasing understanding of the phenomena of bacterial dissociation, it became recognized that the forms originally described as O correspond to those described as S in other species, and the expression O substance is now used to designate the surface component of the S form. It is this substance which is lost by the cell when it dissociates from the S to the R phase.

Flagella, capsules and other types of surface components can be lost independently of each other or in any combination and cellular components other than those located at the surface can also undergo loss variation. All these variations are naturally reflected in the ability to elicit the production of antibody. A bacterial dissociant which has lost one of its "antigens" fails to elicit the production of the corresponding antibody. As we shall see, this fact is of considerable importance in the selection of the state of the bacterial culture to be used for immunization. Moreover, it is precisely by comparing the behavior of different variants of one given culture in antisera prepared against each one of these different variants that one can recognize the presence or absence of cellular constituents which would not have been detectable by ordinary cytologic technics. The immunochemical analysis of bacterial dissociation thus gives a cytochemical basis to the problems of pathogenesis and immunity. The phenomena of variation in antigenic structure have been recently reviewed by Braun (1947) and Dubos (1945, 1946).

#### MECHANISMS OF VARIATIONS

Certain authors believe that many types of variation are specifically induced by the environment, and describe under the name of adaptation or training the process by which a bacterial culture becomes able to attack a new substrate added to the medium, to multiply in the presence of an agent which was bacteriostatic or bactericidal for the parent culture, to grow in the absence of a nutrient originally required, to increase its virulence for a given host by repeated passage through that host, etc. Changes resulting from the "training" possess the following characteristics: they are often reversible, especially in the early phase of training; they exhibit specificity and are therefore qualitatively determined by the environment; they are quantitatively adapted to the modifying environmental factor (for example the degree of resistance to an antiseptic depends in part on the concentration of the antiseptic to which the culture has been exposed). These facts have served as the basis for the theory that adaptive variations are really induced by the environment which exerts its effect by causing quantitative alteration of the enzymatic constitution of the cell (Hinshelwood, 1946).

On the other hand, there are so many similarities between variations in bacteria and mutations in higher organisms that they strongly suggest a common underlying genetic basis. (1) Many variations in morphologic and physiologic characters occurring in cultures of bacteria are transmissible, unchanged, through numerous generations, and are therefore considered to be stable and heritable. (2) The spontaneous origin of certain stable and heritable variations, independent of the specific treatments used to detect them, has been proven in a few cases. (3) Different characters within a strain may vary independently of one another. (4) Physical and chemical agents



known to be effective in increasing the rates of mutations in higher organisms have similar effects on bacteria (X-rays, ultraviolet, mustard gas). The mutations induced by these agents in bacteria, as in higher organisms, seem to be random and nonspecific. (5) Certain variations in bacteria leading to altered growth requirements and synthetic abilities are close analogs of the biochemical mutations in *Neurospora*, a sexually reproducing micro-organism in which the existence of gene-controlled heredity has been established with certainty (Witkin, 1947).

After mutations have occurred, the forces of selection can change the composition of the bacterial population. Thus, the few lactose-positive mutants which occur spontaneously in normal cultures of *E. coli mutabile* are selectively favored in media containing lactose and eventually outgrow the lactose negative forms. Addition of increasing concentrations of an antibacterial agent to a culture medium forces the selection of the normally occurring mutants endowed with the highest resistance to this agent; similarly, passage of a bacterial culture through the animal body determines the elimination of the forms susceptible to the natural and immune defense mechanisms and brings about an increase in virulence through the selection of the forms most resistant to these forces. Since mucoid, smooth, rough, flagellated and nonflagellated variants can occur spontaneously in a given culture, the frequency of mutation and the comparative rates of growth of the different forms under the conditions of cultivation decides the dissociative phase in which the culture grows under these conditions.\*

\* Addition of an antibody to the medium usually modifies the state of aggregation and, consequently, the rate of growth of the culture phase corresponding to this antibody. This may account for the fact that antisera can often be used for bringing about bacterial dissociation. Although it has often been assumed that the antiserum induces the change, it would, according to this theory, only favor the selection of naturally occurring variant forms.

On the basis of present evidence, therefore, the easiest explanation of bacterial variability appears to reside in the occurrence of mutationlike phenomena which usually remain undetected under ordinary conditions but which are rendered evident when growth in selective media or in the animal environment favors the selection of the mutant forms. Nevertheless, the inadequacy of our knowledge concerning the reproduction processes and the transfer of hereditary characters in bacteria, may make it unwise to take a dogmatic attitude with reference to the mechanisms of variation and adaptation. Recent critical reviews of the subject will be found in Braun (1947), Luria (1947) and Tatum and Lederberg (1947).

Tatum and Lederberg (1947) have presented evidence for the occurrence of character recombination in the bacterium *E. coli*; recombinations of genes controlling several growth factor requirements and resistance to a specific bacteriophage were found. If these discoveries are confirmed, and found applicable to other bacterial species, hybridization and segregation may prove to play an important part in the phenomenon of bacterial variability.

#### INDUCED VARIATIONS AND THE TRANSMUTATION OF BACTERIAL TYPES

As in the case of other cells, it is possible to increase the rate of mutation in bacteria by the action of radiation and by the addition of certain substances—the nitrogen mustards for example—to the medium. Of even greater interest is the fact that it has been possible to induce at will certain specific hereditary transformations of the cell. Thus, when properly selected cultures of nonencapsulated pneumococci are cultivated in media containing adequately prepared extracts of encapsulated pneumococci, they acquire, and transfer to their progeny, the ability to produce the type



specific capsular polysaccharides characteristic of the strain from which the transforming extract was produced; for example a nonencapsulated culture derived from Type II pneumococcus grown in the presence of an extract of encapsulated pneumococci of Type III, can be transformed into a culture which continues to produce the Type III capsular polysaccharide in the absence of the transforming extract. The transforming factor, however, is not the capsular polysaccharide itself but is closely associated with, and may consist solely of, a substance which has the composition and properties of deoxyribonucleic acid. Analogous specifically directed hereditary transformations have been induced in the case of *E. coli* also with a deoxyribonucleic acid fraction. It is of great interest naturally, that deoxyribonucleic acid is one of the characteristic constituents of the nucleus of higher organisms and may participate in the transmission of hereditary processes. The problem of hereditary interaction of one bacterial type with another is reviewed by McCarty (1946) and by Tatum and Lederberg (1947).

## PROBLEMS OF BACTERIAL CLASSIFICATION

### CRITERIA USED IN BACTERIAL CLASSIFICATION

Bacteria comprise a diversified variety of micro-organisms, including exacting heterotrophs which require for multiplication all of the known water-soluble growth factors, and strict autotrophs which can synthesize all their protoplasmic constituents from CO<sub>2</sub> and mineral elements; and ranging from the rugged mycobacteria to the most delicate spirochetes. Whether one considers their metabolic and biologic activities or the chemical nature of their structural components, bacteria appear as an ill-defined biologic group, probably heterogeneous in phylogeny. In higher organisms, natural classification is based chiefly on morpho-

logic characters, particularly those concerned with reproduction. These criteria are not available in bacteria, since binary fission is the only mode of reproduction which has been adequately described. (For an exception to this statement, see page 23.) Among morphologic characters, the shape of the cell, the nature and properties of the cell wall, the location of chromatin material, the presence and location of spores and flagella, the method of cell division, the staining characteristics, are of definite use in taxonomy. It is important to emphasize, however, that description of these characters must correspond to cultures grown under well-defined conditions, since cellular morphology is greatly influenced by the physiologic state of the culture and by many environmental factors. Moreover, if spores or flagella are absent one should always proceed with caution since many species—if not all—can give rise to non-flagellated and nonsporulating variants.

Because of the paucity of morphologic criteria, all systems of bacterial classification make considerable use of metabolic and biologic characteristics (enzymatic equipment, ability to perform certain chemical reactions, immunologic specificity, pathogenicity, etc.). Although these criteria have great practical value, they do not necessarily correspond to the fundamental characteristics which are required for classification on a natural basis (van Niel, 1946). Furthermore, bacterial variation often affects many metabolic and biologic characteristics which can undergo profound and lasting modifications, thus adding to the difficulties of classification. Ideally, therefore, complete characterization of a culture for taxonomic purposes requires the description of all the forms which the culture is potentially capable of assuming. It is certain that in many cases, the same species has been described under different names because it was observed in several variant forms.

## SYSTEMS OF CLASSIFICATION

Despite these difficulties, many attempts have been made to classify bacteria in systems possessing a broad biologic basis. Whereas the original classification schemes (Cohn, Migula, etc.) were based almost exclusively on gross morphologic characters (shape of the cells, presence of spores and flagella), all later systems have, beginning

with Orla Jensen, included an increasing number of physiologic and biochemical criteria. During recent years, most of these systems have been codified in the Manual of Determinative Bacteriology, also known as Bergey's Manual, prepared by the Society of American Bacteriologists. The sixth edition of the manual (Bergey, 1948) is organized according to the following outline classification:

TABLE 8. OUTLINE CLASSIFICATION, BERGEY'S MANUAL

Class *Schizomycetes*

## Order I. Eubacteriales

## Suborder I. Eubacteriineae

## Family I. Nitrobacteriaceae

## Tribe I. Nitrobacterieae

## Genus I. Nitrosomonas

## II. Nitrosococcus

## III. Nitrospira

## IV. Nitrosocystis

## V. Nitrosogloea

## VI. Nitrobacter

## VII. Nitrocystis

## Tribe II. Hydrogenomonadeae

## Genus I. Hydrogenomonas

## Tribe III. Thiobacilleae

## Genus I. Thiobacillus

## Family II. Pseudomonadaceae

## Tribe I. Pseudomonadeae

## Genus I. Pseudomonas

## II. Xanthomonas

## III. Methanomonas

## IV. Acetobacter

## V. Protaminobacter

## VI. Mycoplana

## Tribe II. Spirilleae

## Genus I. Vibrio

## II. Desulfovibrio

## III. Cellvibrio

## IV. Cellfalcicula

## V. Thiospira

## VI. Spirillum

## Family III. Azotobacteriaceae

## Genus I. Azotobacter

## Family IV. Rhizobiaceae

## Genus I. Rhizobium

## II. Agrobacterium

## III. Chromobacterium

## Family V. Micrococcaceae

## Genus I. Micrococcus

## II. Gaffkya

## III. Sarcina

## Family VI. Neisseriaceae

## Genus I. Neisseria

## II. Veillonella

## Family VII. Lactobacteriaceae

## Tribe I. Streptococceae

## Genus I. Diplococcus

## II. Streptococcus

## III. Leuconostoc

## Tribe II. Lactobacilleae

## Genus I. Lactobacillus

## II. Microbacterium

## III. Propionibacterium

## IV. Butyribacterium

## Family VIII. Corynebacteriaceae

## Genus I. Corynebacterium

## II. Listeria

## III. Erysipelothrix

## Family IX. Achromobacteriaceae

## Genus I. Alkaligenes

## II. Achromobacter

## III. Flavobacterium

## Family X. Enterobacteriaceae

## Tribe I. Eschericheae

## Genus I. Escherichia

## II. Aerobacter

## III. Klebsiella

## Tribe II. Erwinae

## Genus I. Erwinia

## Tribe III. Serrateae

## Genus I. Serratia

## Tribe IV. Proteae

## Genus I. Proteus

## Tribe V. Salmonelleae

## Genus I. Salmonella

## II. Shigella

## Family XI. Parvobacteriaceae

## Tribe I. Pasteurelleae

## Genus I. Pasteurella

## II. Malleomyces

## III. Actinobacillus

## Tribe II. Brucelleae

## Genus I. Brucella

## Tribe III. Bacterioideae

## Genus I. Bacteroides

## II. Fusobacterium

## Tribe IV. Hemophileae

## Genus I. Hemophilus

## II. Moraxella

## III. Noguchia

## IV. Dialister

## Family XII. Bacteriaceae

## Genus I. Bacterium

## Family XIII. Bacillaceae

TABLE 8. OUTLINE CLASSIFICATION, BERGEY'S MANUAL (*Continued*)

Genus I. <i>Bacillus</i>	Genus I. <i>Streptomyces</i>
II. <i>Clostridium</i>	II. <i>Micromonospora</i>
Suborder II. <i>Caulobacteriineae</i>	Order III. <i>Chlamydobacterales</i>
Family I. <i>Nevskiaceae</i>	Family I. <i>Chlamydobacteriaceae</i>
Genus I. <i>Nevskia</i>	Genus I. <i>Sphaerotilus</i>
Family II. <i>Gallionellaceae</i>	II. <i>Clonothrix</i>
Genus I. <i>Gallionella</i>	III. <i>Leptothrix</i>
Family III. <i>Caulobacteriaceae</i>	Family II. <i>Crenothricaceae</i>
Genus I. <i>Caulobacter</i>	Genus I. <i>Crenothrix</i>
Family IV. <i>Siderocapsaceae</i>	Family III. <i>Beggiatoaceae</i>
Genus I. <i>Siderocapsa</i>	Genus I. <i>Thiothrix</i>
II. <i>Sideromonas</i>	II. <i>Beggiatoa</i>
Appendix: Family <i>Pasteuriaceae</i>	III. <i>Thiospirillopsis</i>
Genus I. <i>Pasteuria</i>	IV. <i>Thioploca</i>
II. <i>Blastocaulis</i>	Appendix: Family <i>Achromatiaceae</i>
Suborder III. <i>Rhodobacteriineae</i>	Genus I. <i>Achromatium</i>
Family I. <i>Thiorhodaceae</i>	II. <i>Thiovulum</i>
Genus I. <i>Thiosarcina</i>	III. <i>Macromonas</i>
II. <i>Thiopedia</i>	Order IV. <i>Myxobacterales</i>
III. <i>Thiocapsa</i>	Family I. <i>Cytophagaceae</i>
IV. <i>Thiodictyon</i>	Genus I. <i>Cytophaga</i>
V. <i>Thiothece</i>	Family II. <i>Archangiaceae</i>
VI. <i>Thiocystis</i>	Genus I. <i>Archangium</i>
VII. <i>Lamprocystis</i>	II. <i>Stelangium</i>
VIII. <i>Amoebobacter</i>	Family III. <i>Sorangiaceae</i>
IX. <i>Thiopolycoccus</i>	Genus I. <i>Sorangium</i>
X. <i>Thiospirillum</i>	Family IV. <i>Polyangiaceae</i>
XI. <i>Rhodomonas</i>	Genus I. <i>Polyangium</i>
XII. <i>Rhodothece</i>	II. <i>Synangium</i>
XIII. <i>Chromatium</i>	III. <i>Melittangium</i>
Family II. <i>Athiorhodaceae</i>	IV. <i>Podangium</i>
Genus I. <i>Rhodopseudomonas</i>	V. <i>Chondromyces</i>
II. <i>Rhodospirillum</i>	Family V. <i>Myxococcaceae</i>
Family III. <i>Chlorobacteriaceae</i>	Genus I. <i>Myxococcus</i>
Genus I. <i>Chlorobium</i>	II. <i>Chondrococcus</i>
II. <i>Pelodictyon</i>	III. <i>Angiococcus</i>
III. <i>Clathrochloris</i>	IV. <i>Sporocytophaga</i>
IV. <i>Chlorobacterium</i>	Order V. <i>Spirochaetales</i>
V. <i>Chlorochromatium</i>	Family I. <i>Spirochaetaceae</i>
VI. <i>Cylindrogloea</i>	Genus I. <i>Spirochaeta</i>
Order II. <i>Actinomycetales</i>	II. <i>Saprospira</i>
Family I. <i>Mycobacteriaceae</i>	III. <i>Cristispira</i>
Genus I. <i>Mycobacterium</i>	Family II. <i>Treponemataceae</i>
Family II. <i>Actinomycetaceae</i>	Genus I. <i>Borrelia</i>
Genus I. <i>Nocardia</i>	II. <i>Treponema</i>
II. <i>Actinomyces</i>	III. <i>Leptospira</i>
Family III. <i>Streptomycetaceae</i>	

Although Bergey's Manual is coming into increasing use and may ultimately become the internationally accepted handbook on bacterial taxonomy, its basis of classification has not remained unchallenged, and many alternative keys have been proposed in an attempt to bring out more clearly phylogenetic relationships among bacteria (reviewed in van Niel, 1946).

#### CLASSIFICATION OF PATHOGENIC BACTERIA

Of the many species of bacteria which have been recognized, only a few exhibit pathogenic properties. Many of the pathogenic species, on the other hand, exist in a large number of subspecies designated as groups, types, strains, etc. Because of the need for practical methods to differentiate these subspecies, the medical bacteriologist



has developed additional systems of classification for each one of the pathogenic groups. Thus, pathogenic mycobacteria are classified on the basis of host susceptibility as human, bovine, murine and avian strains. Needless to say, this is a most unsatisfactory classification which should be superseded by others based on cultural or immunologic characteristics. The case of the salmonella offers a good illustration of the evolution of bacterial taxonomy. At the beginning, individual pathogenic strains were designated by names recalling the type of disease which they cause, their discoverer, or the place where the organism was isolated. As more and more strains were recognized, the problem of separating and classifying them led to the development of differential media based particularly on fermentation reactions. During recent years, however, the systematic study of the immunologic relationships in this bacterial genus has led to the recognition of certain cellular constituents which can be identified by precise serologic reactions (Kaufman-White scheme based on antigenic analysis). Just as in the animal kingdom, serologic reactions have been used for the identification and classification of erythrocytes on phylogenetic grounds, antigenic analysis will certainly facilitate the natural classification of bacteria. It must be remembered, however, that substances possessing similar immunologic specificities may occur in unrelated microbial groups; for example, polysaccharides giving rise to cross-serologic reactions have been recognized in pneumococci, Friedländer bacilli, salmonella, and yeasts. Immunochemical relationships, therefore, cannot be used as the sole basis of classification.

#### CORRELATION BETWEEN STAINING CHARACTERISTICS AND BIOLOGIC PROPERTIES

As already mentioned, it is possible to divide the bacterial world into three broad groups: Gram positive, Gram negative and

acid fast, on the basis of empirical staining reactions. This division is not absolute since there are intermediate forms which are ill defined in their relation to staining technics. Nevertheless, the classification based on staining properties corresponds to fairly well defined differences in chemical and biologic properties between the three bacterial groups. It appears worth while, therefore, to review briefly some of the characteristics which appear to be correlated with staining properties.

The ability to retain the Gram stain appears to be associated with the presence in the cell of magnesium ribonucleate in loose combination with a protein from which it can be readily separated. Measurements of electrophoretic mobilities and of the ability to retain basic dyes indicate a marked preponderance of acid over basic groups in the Gram-positive bacteria, and have led to the statement that these organisms possess overall isoelectric points ranging from pH 2.5 to 4.0. In general, Gram-positive differ from Gram-negative organisms in their susceptibility toward certain antibacterial agents; for example, the former organisms are usually much more susceptible to the inhibiting effect of basic dyes, anionic detergents, penicillin, gramicidin, bacitracin, subtilin, etc.; there are, naturally, important exceptions to this rule such as the great susceptibility of gonococci and meningococci to penicillin.

The isoelectric points (as defined above) of the Gram-negative bacilli are clustered between pH 4.5 and 5.5, i.e. less on the acid side than in the case of Gram-positive bacteria. When Gram-negative bacilli in the smooth phase are extracted by a variety of reagents (trichloroacetic acid, diethylene glycol, phenol, aqueous pyridine, etc.) they yield in solution phospholipid-polysaccharide-protein complexes known as O antigens and endotoxins. These determine the immunologic specificity of the strain through their polysaccharide component; their toxicity is not readily destroyed by high tem-

peratures. In spite of many attempts, complexes with similar antigenic and toxic properties have not yet been recovered from Gram-positive bacteria. As already mentioned, most Gram-negative bacilli and vibrios are much more resistant to certain antibacterial agents than the Gram-positive species. On the other hand, they are extremely susceptible to the bactericidal effect of specific immune serum in the presence of complement, both in vitro and in vivo.

Acid-fast bacilli are difficult to stain and, once stained, equally difficult to decolorize. These organisms are characterized by extremely high concentrations of a variety of lipids, which may reach up to 30 per cent of the total weight of the cell in the pathogenic species. It is certain that the high lipid concentration is responsible for the hydrophobic character of the cell surface of mycobacteria, and for their peculiar

mode of growth on the surface of aqueous media. It is also known that the different lipids elicit characteristic types of tissue response in the invaded host, playing a dominant part in the histopathologic picture of tuberculosis. As a group, acid-fast bacilli differ from the nonacid-fast species in their behavior toward antibacterial agents. Although they are resistant to certain ordinary antiseptics, they exhibit marked susceptibility to others which are only poorly active against the ordinary nonacid-fast species. Special mention must be made of the striking resistance of tubercle bacilli to strong acids and alkalis, a property which is widely used for the selective elimination from suspected pathologic material of contaminating nonacid-fast bacteria which interfere with the bacteriologic diagnosis of tuberculosis.

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### 3

## Parasitism and Disease

Medicine has been so occupied with the pathology of invasion of the human body by micro-organisms as etiologic agents of specific diseases that it has found it difficult to accept disease as part of the broader biologic phenomenon of parasitism. For their self-maintenance, the various species subsist by their ability to gain from other living organisms the requirements for their growth and reproduction. In this struggle for existence different stages of behavior are encountered, varying from that of the predatory agent, such as man or the anthrax bacillus which attacks and destroys its source of maintenance, to that of the *commensal* organism which survives with or on another without evidence of injury through the association, or that of *symbionts* which presumably are of mutual aid. At present these differences in behavior are all encompassed within the general concept of parasitism, representing stages, in adaptation, of an organism in a competitive environment. Let us go on to quote Theobald Smith:

Parasitism is in a sense a compromise or truce between two living things, accompanied by predatory processes whenever opportunity is offered one or the other party. The universality of parasitism as an offshoot of the predatory habit negatives the position taken by man that it is a pathological phenomenon or a deviation from the normal processes of nature. The pathological manifestations are only incidents in a developing parasitism. As hu-

man beings intent on maintaining man's domination over nature we may regard parasitism as pathological insofar as it becomes a drain upon human resources. . . . Too often it has been assumed that parasitism was abnormal and that it needed only a slight force to re-establish what was believed to be a normal equilibrium without parasitism. On the contrary, biology teaches us that parasitism is a normal phenomenon . . . (Smith, 1934).

It is interesting, however, to observe that a high degree of selection is exhibited in the engaging of a host indicating that the conditions which permit the establishment of a parasite are quite exacting. It suggests the probability that relatively specific forms of nourishment must be available in a host under physical or physiological circumstances which permit the parasite to utilize them to its proper advantage. These influences then account for the fact that most parasites have a limited range of hosts. And some of them with complicated developmental stages actually require a different host for each stage. Moreover, the parasite must be of a nature that can be tolerated by the host cells so as to permit survival of both organisms. The poorly adjusted parasite, with its predatory activities still dominant, rapidly ravages the environment to which it has gained access so as to deplete it, and the excursion terminates in the death of the host and of the parasite; or it induces the mobilization by the host of defense mechanisms which destroy the invader.

However, the efficient parasite, as Swellengrebel (1939) terms it, lives in harmony with its host, nursing upon it, but not to the extent of depleting its vigor, nor in such a way as to induce a reaction upon the part of the host which will disrupt the desired association.

For the perpetuation of a parasitic species, there are four requirements (Smith, 1934). *First, the parasite must gain entrance to the host.* The portals of entry to the human body are essentially limited to the orifices of the body and the integument. The mouth and nose constitute the most important portal since they provide the approaches to the respiratory and alimentary tracts, and to the central nervous system as well as to the lymphatic and blood streams. And, since this is also the gateway to mucous membranes most constantly exposed to gross contamination from the outside through food, fluid, air and direct transfer of infected material, it is in all respects the route of greatest importance in human parasitism.

The genito-urinary tract is a limited portal in that opportunities for admission are somewhat restricted by mores and functions. The conjunctival sac is a potential portal, but in this instance, too, physiologic mechanisms limit the opportunities for gross contamination and lodgment.

The skin presents a wide area on which organisms can alight, but there are few instances in which the unbroken skin readily admits a parasite. Nevertheless, the glandular ducts, hair follicles and wounds offer avenues of approach, and certain animal parasites may actually pierce the skin. In addition, biting insects may readily penetrate the skin, introducing a parasite directly into the lymphatic bed or into the blood stream.

However, it is not sufficient that the parasite enter the body but, in most cases, it must enter the tissues to establish a suitable residence in which it can develop. This is ordinarily accomplished by penetration

of the superficial tissues and transport to locations which satisfy its environmental and nutritional demands. Otherwise, it may be discarded as an inert substance. The capacity to become established in the host constitutes the organism's *infectivity*.

*The second requirement is that of multiplication and adaptation.* It is in the effort to fulfill this need that the biologic contest between the mechanisms of the host and those of the parasite reaches its height and determines the outcome. And it is on this phase that the interest of medicine has been primarily centered. If the invader can establish itself without injury to the host it may become part of the normal flora as a benign, *saprophytic* organism. In many instances, however, injury is created which in turn calls forth a reaction on the part of the injured host; the reaction constitutes *disease*, and the agent is said to be *pathogenic* for that species of host. The severity of the injury created or of the invasion is a measure of the *virulence* of the foreign organism.

To counteract the activities of the organism which are harmful to the host, the latter brings into play its own defensive reactions which are then exerted against the invading parasite so as to limit its capacity to produce the injurious effects or to dislodge it. Since the parasite is also a living, physiologic system, it in turn utilizes its mechanisms of resistance against the attack of the host. The contending forces, offensive and defensive, of host and parasite, continue, then, until an adjusted equilibrium is reached in which the parasite may have been forced to give up its actively aggressive qualities or until the host's defenses have been mobilized to neutralize the noxious activities of the organism as they are produced. The organism may become nontoxic, as with avirulent diphtheria bacilli; pathogenically degraded, as with the pneumococcus which loses its capsule; and the host may become staunchly resistant. Nevertheless, the potential capacities of



either to injure the other are not necessarily destroyed, and the stalemate may be broken if the defenses of either engaged party are not maintained. At other times the parasite may become highly resistant to the unfavorable medium, as is seen in the bacterium which becomes insusceptible to the action of therapeutic drugs through modification in its enzyme system. If the invader cannot be well tolerated by the host, the action may be brief and severe disease, with death to one or the other. In other instances the struggle may be a prolonged series of skirmishes with intervening quiet periods; i.e., chronic disease with exacerbations. The two species of living organisms are maintaining a system of balances and checks upon one another to prevent an undue advantage to the opponent.

Although the parasite may find a suitable individual host in which to survive, the ultimate death of that individual will terminate its capacity to maintain the parasite except in those instances in which dead tissue is utilized. Consequently, a *third requirement for parasitism is that there must be a satisfactory portal of exit from the host by migration or discharge*. In the majority of instances, parasites which enter by way of the respiratory tract find egress through respiratory excretions and parasites of the alimentary tract tend to enter and leave by the alimentary route. Those brought to the host by intermediate insects usually require the insect for release. It is obvious, however, that organisms which invade widely may, because of lodgment in different organs, find other channels for escape. There is, nevertheless, a tendency for one route to be the dominant one in the cycle of a given parasite, and its recognition is important in the understanding of the particular problem, especially in efforts toward control.

*The fourth requirement for perpetuation of parasitism is that there must be an effective mechanism for transmission to new hosts*. It is apparent that, except for or-

ganisms which form resistant stages, such as spores, which can persist, propagation requires relatively prompt parasitization of a new host, whether it serves as an intermediate host of another species for one stage of development or as a single definitive species.

Many agents pass constantly from the infected excreta of one individual to another of the same species so that in the evolution of their parasitic state they become limited to a single host. Consequently, the materials and agencies involved in the escape serve as the materials for transmission to the new subject, as in direct transfer of respiratory, intestinal or urogenital discharges; the indirect transmission through water, milk and food; the transfer by intermediate insect vectors; the direct transfer by handling or ingestion of infected tissues.

Although these patterns tend to become stabilized, they do not of necessity equilibrate at a harmless level. The parasite which has developed a defense against its host may be in position to act more efficiently when it reaches a new, inexperienced individual so that the sequence of lodgment, invasion and multiplication is more readily completed and the resultant history is that of repeated cycles of infection at a level which represents severe disease in the host population and leads to the establishment of a consistent disease picture.

There are in contrast the parasitic systems in which a parasite and its host, either as a species or a community, have become adapted to each other at a level of less active injury and tend to maintain this equilibrium. The introduction of a new species or an inexperienced population into that equilibrium may lead to a sudden migration of the parasite from its customary pathways, attacking the new host with severe results to the latter. Man's rôle in many of the diseases acquired from other animals is purely that of the accidental interloper, as in rabies, plague or brucellosis. Moreover, in the new host the parasite may become

modified so as to change its constitution and antigenic behavior, thus giving rise to new strains and new disease pictures. Theobald Smith has considered these aberrant movements the most important factors in parasitism in its relation to disease. But in aberrant movement the organism does not necessarily adapt well to the new host and, consequently, is not passed in series to other individuals of the species. In its straying to a new host the agent may fail to obtain a proper means of transfer and thus it is lost. Or it may actually stray within its normal host to an unnatural location and thus lose its mechanism for further transmission. It may, however, find the new abode acceptable; the parasite then adds to its host range and is said to have multiple hosts. Infection with *Br. tularensis* is one of the most widespread in animal species, indicating the high degree of its adaptability. It is perhaps one of the most characteristic features of parasitic life that it is constantly seeking new hosts so as to widen its opportunity for implantation and survival.

### BACTERIA AS PARASITES

The two centuries between the discovery by Leeuwenhoek of a new world in microscopic biology and the actual demonstration by Koch that human disease could be caused by bacteria saw slow progress in the acceptance of microbiology into natural science. The functional activities of bacteria were obscure, and even as advanced a thinker as Jacob Henle in 1840 was unwilling to speculate on the exact class of parasites responsible for infectious disease. The development of knowledge of bacteria, of their wide variety, of their anatomy, physiology and biochemistry (Dubos, 1945) has dispelled one of the great reservations as to their inclusion among the parasites: that their processes of invasion, variation and adaptation and the responses of the host to specific components of the bac-

terium were not well known. Their cycles of procedure from man to man or from other species to man have been elucidated. In addition, the recognition that even the disease-producing bacteria, many still predatory in behavior, represent parasites acting at a harmful level has removed the need for considering pathogens as a special biologic class. Representatives of the same species of bacteria will vary extremely in their participation in clinical medicine as they may be mucoid or smooth pathogens, or rough, degraded saprophytes of but potential danger. A great number of bacterial residents of the mucous cavities of the skin and of the intestinal tract have become so completely adapted and accepted that they constitute a normal population or flora, and their association with disease is usually accidental. And studies of nutrition indicate that some of these organisms can participate beneficially to the host in preparation of food stuffs for digestion and absorption or in possibly serving to synthesize essential accessory substances.

### INTRACELLULAR PARASITES

Although bacteria in general tend to take up residence in specific tissue environments, in numerous instances they remain outside the tissue cells and feed upon the secretions and excretions of the area. Some bacteria, however, enter the cells and maintain an intracellular state as an important feature in the disease process; tuberculosis and brucellosis with their chronic illnesses and relapses are illustrative. And the parasite in this position is a much more difficult problem of disease than those which act at the surfaces. It no longer behaves merely as a competitor for a general food supply, but actually lives at the expense of the cell. As one progresses downward in the scale of disease-producing organisms the importance of the intracellular state becomes greater. Thus the rickettsiales maintain their major cycle in the cells of the



animal body as they arrive from their arthropod reservoir. Those of spotted fever occupy the nucleus and those of epidemic typhus are found in the cytoplasm. The agents which are classified as *viruses* have become recognized as obligatory intracellular parasites which can only grow in the living cells. They are thought of as parasites which have degenerated with loss of most functions of independence, retaining only that of reproduction. Energy requirements are met by the diversion of the cell's metabolism to the purposes of the virus. The biologic position of agents classified as viruses is not uniform; the Chlamydozoa of psittacosis and lymphogranuloma venereum are clearly organisms; the agents of the pox diseases are certainly parasites. The nature of the more minute ones is less well established, but the processes of introduction to the host's tissues, their adaptation and mutation all follow the plan of organisms. It has even been suggested that entering an organ the viruses may persist thereafter, thus accounting for continued stimulation of the host's defensive mechanisms. And it is certainly clear that the intracellular viral parasites are more closely related through this requirement than through most other characteristics. The need for a definite cell type for a given virus emphasizes once more the high selectivity of the parasite.

### EXAMPLES IN DISEASE

It becomes apparent that the various phenomena observed in infectious diseases of man find their parallels in the classic concepts of reactions between a parasite and its host. A bacterium is a complex anatomic structure possessing diversified and adaptable physiologic functions which furnish the mechanisms by which it utilizes the food stuffs available in another body for its own survival. That it damages the host is incidental from the point of view of the parasite. The cells of the human body then

respond with physiologic functions with which they have been equipped to meet harmful influences; here again, the behavior of the host may be to some extent incidental to the fact that the harm is derived from a living agent. The significance of the animate organism is that it can dwell in the body and continue to produce the injurious component through its own metabolic activities.

The predominant tendency for pneumococci to infect the respiratory tract, for the organisms of typhoid fever to seek the intestinal lymphatic tissue, for the gonococcus to seek the urinary mucosa and for viruses to injure only specific organs and cells strongly indicates a purposeful selection of a tissue medium and environment which will most readily furnish specific substrates for the maintenance of the respective organisms. Similarly the tendency of these organisms to limit their selection of hosts to man suggests that the human environment furnishes a better and more ready source of the substrate required for the particular agents than do other species. And the inability of many of the agents to cause characteristic disease in other species indicates that those species do not furnish the medium to which the organisms have become accustomed in their evolutionary adaptation. In contrast, organisms such as the streptococcus or the tubercle bacillus may find their requirements in a wide variety of tissues and give rise to highly variable disease pictures involving different organs. Still others may localize in an area but create greatest injury by excretion of toxins which are transported to distant organs. The toxins of *C. diphtheriae* or of *Cl. tetani* are produced by growth of the organism at the site of localization, but the fatal injury is exerted more remotely upon nervous tissue. *Cl. welchii* produces in injured tissue a toxin of known enzymatic behavior, a lecithinase, which advances its progress and penetration; nevertheless, in the environment of the human intestine it



is ordinarily found without accompanying evidence of disease.

It has been pointed out earlier that, in order to maintain itself, an organism may acquire characteristics fitting it to the conditions of the habitat or, in order to evade the attack of the host, it may be forced to give up capacities of aggression. Thus, some of the organisms, such as virulent encapsulated pneumococcus, may survive quietly on the mucous membranes of a carrier host who has mobilized many of his immune reactions, or *S. typhosa* may remain in the gall bladder or intestines in an atmosphere of general immunity which it resists by adaptation to a limited environment. Brucellae may persist in the spleen of a resistant host. But others encounter such strong defenses against specific components of the bacterial cell that the organism actually gives up the component which makes it a pathogenic agent. The pneumococcus in the presence of immune serum may lose its capsule and become an apparently benign agent; under certain conditions it may revert spontaneously to encapsulated forms. Although the latter phenomenon has not been clearly demonstrated in the animal body, it is certainly possible and would explain some of the obscure facts concerning the occurrence of Type I and Type II pneumococcal pneumonia. These adaptive physiologic mechanisms result in distinct modifications of the organism directed toward the maintenance and in the development of different strains or races. It is probably through similar variations that diseases such as the poxes in man, cow, sheep and other hosts, or malaria in a wide range of species, have arisen from a common stem to survive in their selected animal circuits. Or that certain strains of an organism may be pathogenic for animal species which are completely refractory to other types. The manner in which the activities of different micro-organisms determine their behavior in creating pathologic phenomena and how immunity comprises

defenses of the host directed against these individual capacities is discussed more fully in subsequent chapters.

When the characteristics of the agent become comparatively fixed, however, and its cycle of parasitism is well established, the disease tends also to approach a constant pattern with the differences in severity of the clinical reaction to infection determined by variations in virulence of microbic strains and in the experience of the host. We quote Henle on this matter:

Actually the more characteristic a miasmatic-contagious disease is, the more certain it is that it has prevailed in essentially the same form from those times to which historical research extends, and if it has apparently occurred anew anywhere, then it can be plausibly explained that it has either only been transplanted from another place to that one or that circumstances have favored its extension and increased its virulence to such a degree that it attracts more attention than formerly (Henle, 1840).

When a single host is involved this seems to be true as evidenced by the consistent behavior of measles, chickenpox or typhoid fever. But with the increasing invasion by man of new areas so as to encounter numerous examples of parasitism stabilized in lower animals the opportunity for establishing new parasites and new diseases of man is greatly increased.

On the other hand, changes in the habits of man as they relate to habits of personal cleanliness or to the selection and protection of food may, by limiting his experience, reduce the resistance of a population to an infectious agent with the result that the pattern of a disease may change from a common, low-grade infection to one of more severe, epidemic disease of limited extent—as has been suggested of poliomyelitis.

To summarize, the phenomena of infectious disease are manifestations of evolving parasitism, the interplay of two complex biologic systems seeking to maintain themselves in a competitive world. That in this struggle to establish itself in a desirable

host, the parasite causes injury is accidental, although its selection of a host and the site of localization seem purposeful. The activities of the parasite and of the host for and against implantation, respectively, represent the forces of infection and immunity.

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## 4

# Properties of Bacteria Which Enable Them to Cause Disease

### VIRULENCE AND PATHOGENICITY

It is the purpose of this chapter to discuss some of the factors which enable bacteria to cause disease.\* The term *pathogenicity*, as generally used, refers to the capacity of micro-organisms to cause disease, either natural or experimental. Thus, pneumococcus is pathogenic for the mouse although spontaneous pneumococcal infections have never been observed in this animal. Nonetheless, the mouse is extremely susceptible to experimental infection and rapidly succumbs following the injection of only a few pneumococci. In order for disease to occur naturally in a population, pathogenic bacteria must be endowed also with certain attributes which will be discussed under the collective term *communicability*, although it should be borne in mind that factors pertaining to the host in addition to intrinsic properties of the micro-organisms, play a very important part in the transmission of infection from one individual to another. The expression *virulence* has been reserved to imply measurement of pathogenicity. However, it is difficult to adhere strictly to this distinction between pathogenicity and virulence, and

most students of infectious disease have used the words interchangeably. In nature a balance, for which the term "parasitism" may be properly used, is usually maintained between the potentially pathogenic micro-organisms and the normally insusceptible host. When the balance of parasitism is tipped either by an increase in virulence of the micro-organisms or a lowering of the natural resistance of the host, disease results. Bacteria abound in nature. They are present in the water we drink, the food we eat, the air we breathe and almost everything we touch. The nasal mucosa, the mouth, the throat, the intestinal tract and the skin each harbor their characteristic bacterial flora in countless numbers. Why do these highly parasitic organisms fail to produce disease in the normal healthy individual, while other related and apparently indistinguishable strains of the same species cause a severe infectious process when introduced in small numbers? Why do certain organisms cause disease in some individuals and not others of the same population? It is obvious that complete answers to these questions will involve not only a discussion of the aggressive attributes of pathogenic bacteria but also a consideration of host resistance. Moreover, it should be clearly understood at the outset that factors which govern the virulence of one bac-

\* The following general reference books may be cited: Metchnikoff (1905), Smith (1934), Zinsser, Enders and Fothergill (1939), Burnet (1940), Dubos (1945), Topley and Wilson (1946).



terial species in a given host do not necessarily apply to other host-parasite systems. This is best demonstrated by a pair of brief examples.

If washed tetanus spores are introduced into normal healthy tissues of experimental animals, they fail to germinate but instead are rapidly engulfed and eliminated by the phagocytic cells (Fildes, 1927). No disease occurs. If, on the other hand, the spores of *Cl. tetani* are introduced into a wound together with some agent capable of causing local tissue damage or necrosis, germination of spores takes place and growth of the vegetative form of the tetanus bacillus occurs locally. During growth, a highly poisonous and freely diffusible toxic protein, tetanus toxin, is elaborated by the organisms and diffuses into the tissues. Tetanus toxin apparently travels along the peripheral nerves to the anterior horn cells of the central nervous system and after a variable incubation period of from three to ten days, tetanus, a disease which is commonly fatal, occurs. The bacteria themselves are present only at the original site of introduction. All the symptoms of the disease can be reproduced in animals by injection of minute doses of sterile tetanus toxin.

If we now consider what takes place following injection of a few virulent anthrax spores into the tissue of a susceptible animal, a quite different situation obtains. The spores are not taken up by the phagocytic cells, as are tetanus spores, but rapidly germinate, multiply and invade all the tissues. The blood stream becomes loaded with anthrax bacilli, and, after death, the organisms may be found in great numbers in all parts of the body, in striking contrast to the animal with tetanus. On the other hand, no demonstrable toxin in any way analogous to tetanus toxin is elaborated by *B. anthracis*.

Thus, in the one case we are dealing with a fatal disease caused by a toxic metabolic product of an organism which is incapable of multiplying in healthy tissue and in the

other, a highly invasive organism causing death only after enormous multiplication within the living tissues of the host. It is obvious that the mechanisms by which these two organisms cause disease are quite different and that any statement to the effect that the tetanus bacillus is either more or less virulent than the anthrax bacillus has no meaning whatever.

Broadly speaking, most diseases caused by bacteria fall into either one of the two categories illustrated by the preceding examples or into a third category in which the pathogenic organism is both invasive and capable of producing one or more toxins as well.

## ENHANCEMENT OF VIRULENCE

Enhancement of bacterial virulence through animal passage is readily demonstrable in the laboratory. Thus, most hemolytic streptococci of Group A and some pneumococci isolated from man are relatively avirulent for mice on primary isolation. By repeated animal passage, virulence for mice may often be increased from a minimal lethal dose of 100,000 organisms or more to a point where only one or two bacteria suffice to bring about a fatal infection. It seems probable that enhancement of virulence by animal passage is the result of selection of virulent mutants present in the original culture. The virulent mutants may differ from the parent strains in one or more ways, as will be discussed presently. These include an increased toxin-producing capacity, the ability to form a protective capsule and the capacity to produce various other substances including certain enzymes concerned in virulence.

It is commonly believed that during the course of epidemics in man the virulence of the infecting agent increases. While this seems reasonable and may be true, there is little objective evidence to support it. The many factors involved in the spread of an epidemic disease make it almost impossible

to establish that virulence has increased. Perhaps the greatest difficulty lies in the fact that virulence of a strain for laboratory animals is rarely a measure of its virulence for man, and since virulence for man cannot be determined under accurately controlled conditions, any inferences must be based upon epidemiologic analysis. Such analysis has not brought forth proof of increasing bacterial virulence during the course of a human epidemic. To the contrary, it would appear even more likely that a highly virulent and communicable mutant was selected through one means or another *before* the epidemic began. If this were not the case, it is difficult to visualize how an epidemic could start.

A possible exception to the statements in the preceding paragraph is the emergence of sulfonamide fast or resistant mutants of bacteria under circumstances where large numbers of infected persons are treated with these drugs over an extended period of time, especially where treatment is inadequate. Under such circumstances sulfonamide-resistant gonococci and Group A hemolytic streptococci have appeared and partially replaced the sulfonamide-susceptible strains previously encountered as causes of epidemic disease. There is no evidence that the resistant mutants are more virulent for *normal* man than susceptible strains. However, the additional attribute of drug fastness, which enables the fast strains to cause disease in persons treated with sulfonamides can perhaps be termed properly an enhancement of virulence, since sulfonamide-sensitive strains are much less capable of causing disease under the same circumstances.

Virulence is also said to be increased by certain artificial methods, such as injecting bacteria suspended in mucin. Enhancement of virulence by such methods should be regarded as more apparent than real. Meningococci, typhoid bacilli and many other Gram-negative species are quite avirulent for mice and to cause death of these

animals must be injected in relatively enormous doses, often 0.1-1 cubic centimeters of broth culture. The number of living organisms required to bring about a fatal "infection" does not differ markedly from the number of heat-killed bacteria which will cause death on injection into mice. In other words, the bacteria themselves are toxic whether living or dead. If living meningococci or typhoid bacilli are suspended in a viscous protective matrix such as mucin, fatal "infections" result in mice after intraperitoneal injection of only a few bacteria. The small inoculum, protected by a surrounding coating of mucin, presumably multiplies until sufficient bacteria are present to constitute a lethal toxic dose. It is questionable whether this phenomenon should be regarded as a true enhancement of the virulence of the organisms by mucin. It may be added that studies of virulence in laboratory animals using bacteria suspended in mucin, have provided little insight into the mechanisms involved in virulence, and moreover do not measure virulence for man.

Just as the virulence of bacteria may be increased by repeated animal passage, so it may often decrease by continued cultivation on artificial media. The process again appears to be one of selection, in this case, of *less* virulent mutants. Many examples of such loss of virulence on laboratory media following isolation might be given, of which only one will be discussed here. *Cl. septicum*, a strict anaerobe not infrequently associated with wound infections in man, produces a potent exotoxin when cultivated in meat broth following isolation from the infected tissue. If the organisms are transferred a few times in a chemically defined medium which supports luxuriant growth, their capacity to produce this toxin is lost, and at the same time the strain loses its virulence (Bernheimer, 1944). If such cultures are plated on blood agar, the colonies are found to be rough in form as contrasted with the smooth colonies formed by freshly



isolated, virulent, toxin-producing strains. It seems probable that the chemically defined medium is lacking in some growth factor present in animal tissue which is required by the smooth virulent organisms but is not required by the rough mutants. The factor is apparently present in meat broth since the change from smooth to rough occurs more slowly on transfer in this medium.

### DISEASES CAUSED BY TOXIN-PRODUCING BACTERIA

Some diseases will now be considered in which injury to the host can be attributed primarily to specific diffusible toxins elaborated by bacteria whose capacity to invade the tissues is either lacking or is limited. In the simplest case the host need not have any contact whatever with the micro-organism which is responsible for the disease. *Botulism*, a relatively uncommon but frequently fatal type of food poisoning, is an example of such a disease. The etiologic agent in this instance is *Clostridium botulinum*, a spore-forming obligate anaerobe whose natural habitat is the soil. The spores of this anaerobe can survive in inadequately sterilized canned food and germinate there. During growth, *Cl. botulinum* produces a toxic protein which is unusually resistant to the action of proteolytic enzymes and which is capable of causing fatal symptoms in animals and in man when ingested. Pure botulinus toxin, Type A, is among the most powerful poisons known. *Cl. botulinum* is pathogenic for man, therefore, because it can produce a potent toxin which is resistant to the enzymes of the intestinal tract and is absorbed following ingestion. The organism itself never gains access to the tissues. We may speak of virulence of *Cl. botulinum* simply in terms of the yield of toxin produced by a given number of bacteria grown under defined conditions.

The pathogenesis of tetanus is more complex than botulism because tetanus spores

must actually lodge in the body. However, germination and multiplication of the organisms can take place only in dead or damaged tissue in an anaerobic environment. Under these conditions tetanus toxin is produced and passes along the peripheral nerves to reach the anterior horn cells. It is probable that different strains vary in virulence according to the amount of tetanus toxin which a given number of organisms are capable of producing under conditions simulating those existing in a deep lacerated wound.

Despite the fact that the diphtheria bacillus is a classic example of an organism which is pathogenic primarily because of its ability to produce a powerful exotoxin, the factors which determine the virulence of different strains of the organism still furnish a subject for controversy. In diphtheria, the bacteria are found growing in a so-called pseudomembrane, which is generally located in the nose or throat. The organisms themselves are almost never found away from the local lesion, but evidence of damage caused by diphtheria toxin may be found in all the organs of the body. Since the lesions in diphtheria, apart from those caused by mechanical obstruction by the membrane, seem to be due almost exclusively to the toxin, it is tempting to conclude that any given strain is virulent in proportion to the amount of toxin which it is capable of producing. Early attempts to demonstrate an increased toxin-producing capacity in culture media by highly virulent strains of the diphtheria bacillus were unsuccessful, however. Indeed, organisms isolated from relatively mild cases of diphtheria often produced many times as much toxin on laboratory media as did strains from severe cases. The early failure to obtain any correlation between toxigenicity and virulence led to a search for some other more potent toxin produced by highly virulent strains. However, although there are many serologic and biochemical differences between strains, all virulent



diphtheria bacilli produce an identical toxin.

Investigations carried out during the epidemics of diphtheria which occurred in England in the late 1920's and early 1930's lead to the demonstration that two markedly different types of diphtheria bacilli designated *mitis* and *gravis* could be differentiated on the basis of certain morphologic and biochemical properties (McLeod, 1943). *Mitis* strains are generally but not always associated with mild cases of diphtheria, while outbreaks of clinically more severe cases are most often caused by *gravis* strains or a third type, *intermedius*. A great many attempts have been made to find out why *gravis* strains are more virulent than are *mitis*. It has been commonly asserted that the virulent types are more "invasive" than are strains of low virulence (Burnet, 1943). There is some evidence that *gravis* strains may produce a second toxin which aids in the spread of the infection in the throat and aids the diffusion of the classic toxin into the tissues (O'Meara, 1940). The extent to which invasion of the tissues by the diphtheria bacillus plays a role in the pathogenesis of diphtheria still remains undetermined.

Mueller (1941) has pointed out that the early attempts to relate toxin-producing capacity with virulence of different strains of the diphtheria bacillus were not carried out under conditions simulating those in the host. In the laboratory it has been shown that maximum yields of toxin are obtained only when the iron concentration in the culture medium is far below that in body tissues (Pappenheimer and Johnson, 1936) or in a diphtheritic membrane. Mueller tested several strains in the presence of an iron concentration comparable to that found in a diphtheritic membrane. He observed that a *gravis* strain isolated from an outbreak of clinically severe diphtheria produced several times as much toxin as did the *mitis* strains tested under these conditions.

The theory that virulence of the diphtheria bacillus depends on the toxin-producing capacity of the organism under the conditions of the body is an attractive one because of its simplicity. Other factors, however, must play a part even in the completely susceptible host. Many of these factors are extremely difficult to evaluate. It seems likely, for example, that the size of the infective dose may well influence the extent of the lesion and therefore the clinical severity. Moreover, animal experiments have shown that the minimum lethal dose of toxin is a function of body weight. Thus a strain of given virulence might easily cause a clinically more severe epidemic of diphtheria among a group of younger school children than in an army camp even if both groups are completely susceptible. In actual practice, the problem is further complicated by the fact that a very large proportion of the population possess some degree of immunity to diphtheria.

One further example of the relation of the toxic products of an organism to the disease which it produces will be considered here. Gas gangrene, a rapidly spreading and often fatal infection of contaminated wounds and compound fractures, may be caused by a number of anaerobic, spore-forming bacterial species whose normal habitat is the soil or the intestinal tract of man and animals. The most frequently occurring organism is *Clostridium welchii*. Gas gangrene differs from the infections which have just been considered in that the lesion spreads rapidly and is not confined to the initial point of entry of the bacteria. Like *Cl. tetani*, the Welch bacillus is capable of multiplying only in dead, necrotic tissue under anaerobic conditions. *Cl. welchii*, however, produces not one, but several toxic substances which rapidly cause the death of the host cells locally and aid in the spread of the infection. The most important of these toxins is an enzyme which hydrolyses lecithin. This lecithinase is hemolytic, dermonecrotic and if injected in a suffi-

ciently high dose is lethal to laboratory animals. In addition to lecithinase, virulent strains of *Cl. welchii* elaborate an extracellular proteinase termed "collagenase," which is capable of depriving muscles of their reticular scaffolding in vivo. The organism also produces hyaluronidase, an enzyme which hydrolyses the hyaluronic acid found in the tissue spaces. It is probable that hyaluronidase and collagenase enable the lecithinase to diffuse more readily through the tissues and cause more extensive necrosis. The organisms then spread into the damaged area where more toxins are produced which by their actions pave the way for a progressive process. Lecithinase is the most important of the toxins produced by *Cl. welchii* since antitoxin specifically directed against this toxic enzyme can prevent the disease in animals whereas antibodies to the hyaluronidase or the collagenase are not protective.

## CHEMISTRY AND PHARMACOLOGY OF BACTERIAL TOXINS

### CLASSIC EXOTOXINS OF GRAM-POSITIVE BACTERIA

Several examples of diffusible toxins produced by Gram-positive bacteria have been discussed. These substances have been called "exotoxins" since they may be found in filtrates from growing organisms exhibiting no visible evidence of autolysis. The term is retained here although it appears quite certain that exotoxins are synthesized within the bacterial cell and in many cases, as with *Cl. botulinum* and *Cl. tetani*, the yield of toxin is greatly increased following autolysis. Exotoxins are characteristic of Gram-positive bacteria and are produced by many pathogenic species. Gram-negative bacteria, on the other hand, produce endotoxins, which are intimately associated with the structural integrity of the cells and are not liberated into the medium unless autolysis has occurred.

Within the last few years the toxins produced by *Cor. diphtheriae*, *Cl. tetani* and *Cl. botulinum*, Type A have been isolated as highly purified heat-labile proteins. Botulinus, Type A (Lamanna et al., 1946; Abrams et al., 1946) and tetanus toxin (Pillemer et al., 1946) have been obtained in crystalline form. These toxic proteins are among the most powerful poisons known. One milligram of either crystalline tetanus or botulinus toxin is sufficient to kill more than 1,000 tons of guinea pig, and it may easily be calculated that the minimum lethal dose (M.L.D.) of botulinus toxin for the mouse is only 20,000,000 molecules. There is a great variation in susceptibility of different animal species to the action of these bacterial toxins (Metchnikoff, 1905). The human, horse and guinea pig show extreme susceptibility to all three toxins. The mouse and rat, on the other hand, are only about 1/1,000th as sensitive as the guinea pig to diphtheria toxin. The dog, while sensitive to diphtheria toxin, is extremely resistant to the action of botulinus and tetanus toxins. Cold-blooded animals appear to be completely resistant to large doses of all three toxins with the interesting exceptions of the frog and certain lizards. While injection of tetanus toxin into these amphibia has no effect at low temperatures, symptoms of tetanus are produced if the animals are kept at temperatures above 20° C. The toxicity of diphtheria, tetanus and botulinus toxin, Type A, for the guinea pig and the mouse is given in Table 9.

Chemical analysis of bacterial toxins has revealed no information to explain their extreme toxicity. Crystalline botulinus toxin has been most carefully analysed and the molecule completely accounted for in terms of its 19 constituent amino acids (Buehler et al., 1947). No unusual chemical groupings which can in any way account for the extreme toxicity of botulinus or of any of the other bacterial toxins have been discovered. Their toxicity appears to reside in the spatial configuration of amino acids within the



PATHOGENIC PROPERTIES OF BACTERIA

TABLE 9. TOXICITY OF PURIFIED BACTERIAL TOXINS \*

ANIMAL	TOXICITY (MLD PER KILO ANIMAL PER MG. TOXIN)		
	DIPHTHERIA TOXIN	TETANUS TOXIN	BOTULINUS TOXIN
Guinea pig	3500	1,200,000	1,200,000
Mouse.....	3.5	200,000	620,000

\* For the sake of comparison, the toxicity has been expressed as minimal lethal dose per kilo of animal per mg. of toxin. The toxicity of tetanus and botulinus toxins for guinea pig was calculated from the ratio of their toxicity for the guinea pig to that for the mouse as given by von Lingelsheim (1912-13) and Meyer (1928) respectively. Similarly the toxicity of diphtheria toxin for the mouse was calculated from the data of Jungeblut (1927).

intact protein molecule, and any method which denatures or in any way alters the protein results in a simultaneous loss in toxicity. Table 10 shows a comparison of some of the chemical and physical properties of diphtheria and botulinus toxin, Type A, with those of a nontoxic protein, human serum albumin.

All three toxins described above are rapidly destroyed by heat (60° C.). Diphtheria and tetanus toxins are unstable in acid solution (the former is denatured below pH 5.6) and both are destroyed by proteolytic enzymes. Botulinus toxin is more stable in acid solution and is not readily attacked by most proteolytic enzymes. It is the only one of the three toxins which is toxic *per os*.

TOXOID FORMATION

A variety of agents react with bacterial toxins causing irreversible loss of toxicity without loss of antigenicity or power to combine with antitoxin. All of these reagents apparently attack free amino groups and include iodine, ketene (an acetylating agent) and diazonium salts. The most com-

TABLE 10. PROPERTIES OF BACTERIAL EXOTOXINS

	DIPH- THERIA TOXIN *	BOTULI- NUS TOXIN, TYPE A †	HORSE SERUM ALBU- MIN ‡
Nitrogen (per cent)	16.0	16.3	15.9
Sulfur.....	0.75	0.44	1.03
Phosphorus	<0.05	0.05	Negative
Amino-nitrogen...	0.98	0.77	..
Cystine.....	..	0.53	5.7
Methionine.....	..	1.06	..
Tyrosine.....	9.5	13.5	4.8
Tryptophane.....	1.4	1.86	0.53
Arginine.....	3.8	4.62	4.9
Histidine.....	2.4	1.03	3.4
Lysine.....	5.3	7.74	13.2
Iso-electric point..	4.1	5.6	4.9
Sedimentation con- stant.....	4.6 S	17.3 S	4.46 S
Diffusion con- stant × 10 <sup>7</sup> ....	6.0	2.14	6.1
Frictional coeffi- cient f/f <sub>0</sub> .....	1.22	1.76	1.27
Molecular weight.	72,000	900,000	70,000

\* Eaton (1936); Pappenheimer (1937, 1942).

† Compiled from data of Abrams et al. (1946); Lammanna et al. (1946); Buehler et al. (1947); Kegeles (1946); Putnam et al. (1946).

‡ Brand (1946).

monly used reagent, however, is dilute formaldehyde. It was discovered by Glenny and Hopkins and by Ramon that crude diphtheria toxin could be completely detoxified by treatment with dilute formalin (0.4-0.5%) at slightly alkaline pH. The reaction is usually complete after three or four weeks at 37° C. The detoxified product termed *anatoxine* by Ramon (1928) and *toxoid* by Glenny and Hopkins (1923) retains its immunologic specificity and antigenic properties. Tetanus and botulinus toxoids may be prepared in a similar manner by treatment of the corresponding toxins with formalin. Diphtheria and tetanus toxoid are used on a large scale for active immunization of man against diphtheria and tetanus.

The change from toxin to toxoid is a



property common to most bacterial toxins and is a process which can occur spontaneously to a certain extent even at low temperatures. This spontaneous detoxification without parallel loss in immunologic combining power was first observed by Paul Ehrlich (1903) who was, in fact, the first to use the term "toxoid."

#### PHARMACOLOGIC ACTION OF THE CLASSIC EXOTOXINS

The pharmacologic action of each of the three toxins which have been discussed is different. Thus diphtheria toxin is capable of causing damage to almost all types of cells in the susceptible animal including skin, muscle, liver, adrenals and nerve. Tetanus and botulinus toxins, on the other hand, appear to act only on the nervous system. It is generally supposed that tetanus toxin acts upon the anterior horn cells of the central nervous system with resulting characteristic tetanic muscular spasms. Botulinus toxin, however, appears to act on the myoneural junctions and causes paralysis of the nerve endings. Death from botulism is due to respiratory paralysis. Despite the fact that characteristic symptoms of diphtheria, botulinus and tetanus intoxication are distinct and characteristic for each toxin, there are certain similarities in their actions which deserve mention. Each of these toxins is capable of causing injury to tissues when administered in very small doses, and it seems likely that only a single molecule per cell is capable of exciting a maximum effect. When small doses are injected there is a characteristic latent period lasting from 24 to 72 hours or even longer, during which time no untoward symptoms of any kind can be observed. In the case of tetanus toxin, the classic work of Marie and Morax (1902) and of Meyer and Ransome (1903) has indicated that the incubation period is in large measure due to the time required for the toxin to travel from the nerve endings

to the central nervous system via the axis cylinders. Thus symptoms of tetanus appear much more rapidly when the toxin is injected directly into the anterior portion of the spinal cord than when injected into the extremities. The latent period also varies with the size of the animal, being shorter the smaller the animal. In the case of diphtheria toxin, nothing is known of what occurs during the latent period. If 1 M.L.D. of diphtheria toxin is injected into a guinea pig, no symptoms or pathologic changes whatsoever can be observed for at least 18 or 24 hours, yet death with the characteristic lesions occurs in four or five days. One M.L.D. of toxin is completely neutralized by about  $\frac{1}{50}$ th unit of antitoxin if mixed before injection. However, even several thousand units of antitoxin fail to save guinea pigs which have been injected only a few minutes previously with 1 M.L.D. of toxin. The fact that the toxin undergoes an irreversible reaction with the tissues within the first few minutes after its injection has an important bearing on its mode of action as well as implications regarding the use of antitoxin in treatment. Diphtheria antitoxin, therefore, is probably effective therapeutically only when administered before the host has absorbed a minimum lethal dose of toxin. It is fortunate that most strains of diphtheria bacilli produce only small amounts of toxin at a relatively slow rate under the conditions existing in the throat so that symptoms of diphtheria intoxication appear before a lethal dose has been absorbed. For these reasons, antitoxin is an effective therapeutic agent when administered early in the disease.

As stated above little is known of what occurs during the latent period and nothing is known of the primary reaction which irreversibly binds toxin to the tissues soon after injection. Even if enormous doses of toxin are administered, the latent period is never completely abolished.

We have considered the properties of diphtheria, tetanus and botulinus Type A

toxins in some detail because they have been most thoroughly studied and have been isolated in a highly purified state. Other bacterial exotoxins are known, but have not been so extensively investigated. These include various hemolysins, scarlatinal or erythrogenic toxin and others.

#### EXTRACELLULAR ENZYMES OF GRAM-POSITIVE BACTERIA WHICH AFFECT THE COURSE OF IN- FECTION

In addition to the classic exotoxins, bacteria produce extracellular enzymes and other substances, some of which are toxic or otherwise play a role in the infectious process.

##### LECITHINASE OF *Cl. Welchii*

Cultures of *Cl. welchii* produce a toxin which is lethal for mice and other laboratory animals. Under suitable conditions culture filtrates may contain as much as 300 or 400 mouse M.L.D. of this lethal or  $\alpha$ -toxin. It has recently been demonstrated that  $\alpha$ -toxin is a lecithinase which, when activated by calcium ions, brings about the hydrolysis of lecithin to phosphorylcholine and a diglyceride (MacFarlane and Knight, 1942). The enzyme differs from the toxic lecithinases of snake venoms in that the latter are esterases which split lecithin to an unsaturated fatty acid and lysolecithin. Lysolecithin is itself toxic and hemolytic. The lecithinase of *Cl. welchii* causes rapid lysis of red blood cells and necrosis of other types of cells. Its action is much more rapid than that of the classic exotoxins which have been described, and there is no appreciable latent period between injection and onset of symptoms. The enzyme activity is destroyed by formalin with production of toxoid. The lecithinase activity is neutralized by specific antitoxin.

##### EXTRACELLULAR PROTEINASES

In addition to lecithinase, culture filtrates of *Cl. welchii* contain a potent proteolytic enzyme which has been termed *collagenase* that is capable of disintegrating muscle tissue of laboratory animals in vivo by decomposing the reticular scaffolding. This enzyme is also neutralized by an anticollagenase present in commercial *Cl. welchii* antitoxin. Collagenase may be responsible for the pulping of muscle often observed in human gas gangrene (MacFarlane and MacLennon, 1945).

Other Clostridia produce powerful proteinases, but their rôle in pathogenesis is still somewhat uncertain. An interesting proteolytic enzyme is that produced by certain strains of Group A hemolytic streptococci. This extracellular proteinase is of the papain type since it is activated by KCN and sulfhydryl compounds. The enzyme rapidly destroys the M protein of Group A hemolytic streptococci, even on living cells, and strains which produce it are avirulent for mice. Enhancement of virulence by mouse passage is accompanied by a loss of capacity to produce the proteinase (Elliott, 1945).

Pathogenic strains of staphylococci produce an active substance known as coagulase, probably an enzyme, which acts in conjunction with a factor present in the serum of certain species to bring about the coagulation of fibrinogen. Coagulase is responsible presumably for the walling off of staphylococcal lesions. In addition, it apparently causes a deposition of fibrin on the surface of the organisms, thereby forming a sort of capsule which protects them from phagocytosis (Hale and Smith, 1945). There is excellent correlation between the capacity to produce coagulase and the pathogenicity of strains of staphylococci.

##### STREPTOKINASE OR FIBRINOLYSIN

Most strains of hemolytic streptococci belonging to Group A, as well as some



Group C and Group G strains produce an extracellular heat-stable protein which brings about the dissolution of fibrin clots. This property of hemolytic streptococci was first described by Tillett and Garner (1933) who termed the substance "fibrinolysin" and showed that it exhibited a remarkable specificity for human fibrin clots. Most animal fibrin clots are relatively resistant to lysis. Further investigation of this interesting substance has shown it to be a kinase which activates a proteolytic enzyme normally present in plasma in an inactive form (Christensen and MacLeod, 1945). Its action is analogous to the activation of trypsinogen to trypsin by enterokinase. Because of these properties, *streptokinase* appears to be a more suitable name than fibrinolysin for the substance discovered by Tillett and Garner. Streptokinase is extraordinarily active in bringing about the dissolution of human fibrin clots. Only about 0.05 microgram of purified preparations is required to cause lysis of a standard human clot within a few minutes. The exudates occurring in streptococcal infections such as peritonitis or empyema are thin and watery and are characterized by the absence of fibrin, doubtless due to the presence of streptokinase. Once specific antibody, antistreptokinase, appears in the blood stream, fibrin appears in the exudates. Streptokinase is not known to be toxic and excepting its role in causing the characteristic exudates little is known of the part which it plays in the pathogenesis of streptococcal infections.

#### HYALURONIDASES

Hyaluronic acid, a viscous high molecular weight polysaccharide acid is present as a constituent of the intercellular ground substance of many tissues. A great many bacteria produce enzymes which are capable of hydrolyzing hyaluronic acid. Hyaluronidases are often adaptive enzymes, i.e. they are only formed in the presence of the substrate, and the hyaluronidases produced by

various bacteria differ in their action on hyaluronic acid. Hydrolysis of hyaluronic acid by *Cl. welchii*, for example, yields different products from those formed through the action of streptococcal hyaluronidase (Rogers, 1946). Injection of hyaluronidase into a tissue increases permeability and allows more rapid diffusion or spreading of injected solutions or of suspensions of India ink and bacteria (Duran-Reynals, 1942). It seems reasonable to suppose that hyaluronidase plays some part at least in enabling bacteria to invade the tissues and to increase the rate of diffusion of toxic products into the tissues of the host. While hyaluronidase production seems to be a property of many pathogenic Gram-positive bacteria, including staphylococci, pneumococci and various Clostridia, in addition to streptococci and *Cl. welchii* mentioned above, no definite correlation has been established between "invasiveness" and hyaluronidase production.

#### BACTERIAL HEMOLYSINS AND CYTOLYSINS

A great many bacteria produce substances which cause lysis of red blood cells and probably of other types of cells as well. In many cases these hemolysins appear to be concerned with virulence and may contribute to the "invasive" properties of some pathogenic species. The hemolysins vary greatly in their properties from one species to another. Indeed, a single strain may be capable of producing two or more hemolysins which differ from one another in their chemical properties and in the mechanism by which they bring about lysis of red cells. Group A hemolytic streptococci commonly produce at least two hemolysins termed streptolysin O and streptolysin S. Streptolysin O is a relatively labile protein which may be oxidized by atmospheric oxygen. The inactive oxidized hemolysin can be reactivated by means of reducing agents such as sulfhydryl-containing compounds or sodium hydrosulfite. Purified preparations of



streptolysin O contain relatively large amounts of sulfur. They are lethal for mice in doses of about 0.1 mg. In contrast to some other hemolytic toxins, streptolysin O does not cause death of animals by intravascular hemolysis. Its hemolytic activity is neutralized by small amounts of cholesterol as well as by specific antisera. Oxygen-labile hemolysins, or O hemolysins, closely related to streptolysin O are produced by other organisms including *Cl. tetani* (tetanolysin), *Cl. welchii* ( $\theta$  toxin) and pneumococcus (pneumolysin). Antisera prepared by immunizing animals with any one of these O hemolysins will neutralize the action of all of them although such anti-hemolytic sera are most active against the homologous lysin.

Streptolysin S may be found in culture filtrates of hemolytic streptococci grown under certain conditions or may be produced when the organisms are incubated with serum. It is not reversibly autooxidizable and is not neutralized by cholesterol. Antisera against streptolysin S are difficult to prepare. They do not neutralize streptolysin O.

Recent studies on hemolysins (Herbert and Todd, 1941; Bernheimer, 1947) have suggested that the bacterial hemolysins are probably enzymes. In only one instance, however, is the substrate for a bacterial hemolysin known, namely that for the lethal toxin or lecithinase produced by *Cl. welchii*. Mice injected with relatively small lethal doses of lecithinase may show complete intravascular hemolysis on autopsy. As mentioned above, *Cl. welchii* produces, in addition to this hemolytic lecithinase, an O hemolysin which has been called  $\theta$  toxin.

The action of bacterial hemolysins is not restricted to the lysis of red blood cells. Thus it seems probable that the *leucocidins* produced by hemolytic streptococci and pneumococci are in reality identical with the O hemolysins produced by these strains. These *leucocidins*, which bring about the

destruction of leucocytes, are reversibly inactivated by oxygen in the same manner as O hemolysins. Further evidence that O hemolysins can affect still other types of cells is suggested by the action of streptolysin O on the isolated frog's heart (Bernheimer and Cantoni, 1946). When the frog's heart is perfused with Ringer's solution containing streptolysin O no apparent effect is noted. However, if the heart is now washed with Ringer's solution and perfused a second time a few minutes later with a very small dose of streptolysin, it undergoes complete systolic contraction. The first dose of hemolysin sensitizes the heart to a second application of the toxin. It has been demonstrated (Cantoni and Bernheimer, 1946) that the initial treatment with streptolysin brings about the release of an inhibitor from the heart. This inhibitor is capable of neutralizing several times the dose required to bring about its release from the frog heart and also is capable of neutralizing the lethal action of streptolysin O for mice.

## ENDOTOXINS OF GRAM-NEGATIVE BACTERIA

Most of the toxins and enzymes that have been described up to this point are released into the extracellular culture fluid by growing Gram-positive bacteria. In the few isolated cases where toxic proteins or enzymes have been found in culture filtrates of Gram-negative organisms, their presence can be definitely ascribed to autolysis. On the other hand, thoroughly washed suspensions of most Gram-negative species, even nonpathogenic ones, are highly toxic. The toxicity of Gram-negative cells is due to characteristic endotoxins or O antigens located at or near the cell surface (Dubos, 1945). Various methods have been employed to extract the toxic O antigens from the cells, using such reagents as trichloroacetic acid, ethylene gly-

col, 90 per cent phenol, pyridine, formamide, etc., and purified endotoxins have been isolated from the extracts. The complete toxic O antigen has been shown to be a complex composed of phospholipid, carbohydrate and protein. Purified preparations of endotoxins are lethal for the mouse in amounts between 0.1 and 1 mg. (Boivin et al., 1933, 1935; Raistrick and Topley, 1934; Morgan and Partridge, 1940; Goebel et al., 1945).

By suitable methods of hydrolysis the complete toxic antigens may be split into a toxic but nonantigenic phospholipid-containing polysaccharide, or to nontoxic nonantigenic phospholipid, carbohydrate, and protein or polypeptide fractions. While not itself antigenic, this protein fraction possesses a remarkable capacity to combine with, and render antigenic, a wide variety of carbohydrates including not only the lipid-free carbohydrates from other Gram-negative species or strains but also gum arabic, agar and the human blood Group A polysaccharide. Such artificial conjugates of degraded protein from the O antigens of Gram-negative cells are powerful antigens, the specificity of which is determined by the carbohydrate component (Morgan, 1943).

Injection of small amounts of endotoxins of Gram-negative bacilli into animals or man results in fever, a sharp rise in blood sugar and fall in inorganic blood phosphorus. The severe local reactions and generalized fever which commonly follow injection of Gram-negative bacterial vaccines are due to their toxic O antigens. It seems probable that pyrogenic reactions following intravenous injection of saline, glucose or therapeutic serums have been frequently caused by the use of water in which growth of Gram-negative organisms had taken place. For this reason great care is now taken that all solutions for intravenous injection are made up in freshly distilled water.

## THE INVASIVENESS OF BACTERIA

An earlier section of this chapter deals with bacteria which are pathogenic chiefly because of their capacity to produce so-called exotoxins that are liberated from the organisms during growth. The essential characters of the diseases caused by these toxigenic bacteria can be reproduced in experimental animals by injection of the toxin itself. With these as well as with other primarily toxigenic species, the capacity to invade and multiply in the living tissues of the host is either weak or entirely absent.

At the other end of the scale from the primarily toxigenic species are those which produce disease because of their invasive capacities, without evidence that exotoxins are involved. Examples of invasive organisms are pneumococcus, the anthrax bacillus, Friedländer's bacillus, *Hemophilus influenzae* and meningococcus. Occupying an intermediate position between the primarily toxigenic and the invasive species are bacteria that produce exotoxins and are also invasive, such as the streptococci and staphylococci. The lesions caused by them reflect this combination of properties.

Invasive bacteria possess one or more surface components which are of primary importance for their pathogenicity, probably because they interfere with phagocytosis. In many instances this surface component is composed of highly polymerized polysaccharides as in the case of pneumococcus, Friedländer's bacillus, *H. influenzae* and others; in the anthrax bacillus it is a polypeptide composed of d-glutamic acid units (Ivanovics and Bruckner, 1938), and in Group A streptococci a surface protein and an acidic polysaccharide, hyaluronic acid, are both concerned in invasiveness. None of these surface components appears to have toxic properties; nonetheless they are of importance in protecting the organisms from the defense mechanisms of the host. Indeed, in the case of Group A strep-



tococci, the hyaluronic acid forming the capsule is very similar to, if not identical with the hyaluronic acid present in the ground substance of normal tissues and found also in the vitreous humor of the eye and Wharton's jelly of the umbilical cord (Kendall, Heidelberger and Dawson, 1937). However, this nonantigenic capsular material appears to exert a protective effect on Group A streptococci. The studies of Hale and Smith (1945) have shown that another normal body constituent, fibrin, deposited on the surface of staphylococci which produce coagulase, protects the organisms from phagocytosis. It is apparent, therefore, that the protection afforded certain invasive micro-organisms by surface components need not depend upon any toxic action, and as in the case of Group A streptococci and staphylococci, these components may be also constituents of the host.

It has been customary to refer to the outermost layer of certain bacteria as a capsule. For the sake of convenience, the term will be retained in the present discussion, but it should be noted that the capsule of bacteria is in no sense the analog of the continuous capsular membrane or sheath which encloses the cysts of various protozoan parasites or contains the seeds of many plants. It is rather a layer of highly polymerized, viscous material, commonly polysaccharide in nature, though not always, which is produced by the cells, sticks to the cell surface and also diffuses into the medium. It is not a limiting membrane.

The surface component present on Gram-negative bacteria, in contrast to those mentioned above, has well-marked toxic properties. This endotoxic component, known as the O, or somatic, antigen, is composed of phospholipid, carbohydrate and protein, with immunologic specificity residing in the carbohydrate fraction. Attempts to isolate and characterize the toxin have not been successful.

#### NONTOXIC SURFACE COMPONENTS AND BACTERIAL VIRULENCE

In the case of virulence of pneumococcus the part played by the polysaccharide capsule has been demonstrated by a variety of independent methods. Noncapsulated variants of pneumococcus appear in cultures grown in the presence of specific immune serum. These noncapsulated variants are avirulent. Additional evidence has been obtained by showing that the purified type specific polysaccharides can be used to absorb the protective antibodies from anti-pneumococcal serum. Moreover, on injecting the polysaccharides themselves into certain animal species, including man, antibodies are formed which protect against infection. An additional line of evidence for the prime importance of the pneumococcal capsule in virulence is provided by the studies of Dubos and Avery on an adaptive enzyme which hydrolyzes the capsular polysaccharide of pneumococcus Type III (Dubos, 1939-40). This enzyme, prepared from a bacterium found in soil, hydrolyzes the Type III polysaccharide even when the latter is present on the surface of the living pneumococcal cells and by so doing destroys the pathogenicity of Type III pneumococcus without affecting the viability of the organisms.

The part played by the pneumococcal capsule in virulence can be shown plainly by phagocytosis experiments in vitro. Encapsulated pneumococci are taken up very slowly by the leukocytes, whereas nonencapsulated forms are rapidly engulfed. If specific antiserum is added to the encapsulated forms they too become quickly phagocytosed and digested. It is clear, therefore, that the pneumococcal capsule acts because it is antiphagocytic, and that specific antiserum neutralizes its antiphagocytic effect. The isolated purified polysaccharides composing it are nontoxic to animals even upon injection of very large doses.



Possession of a capsule does not mean, however, that organisms possessing such a surface structure are pathogenic. On the contrary, most species of bacteria are encapsulated, but only a relatively small number are virulent for man or animals. It is apparent, therefore, that factors in addition to the capsule must play an important part also. The role of such factors can be shown within a single species such as pneumococcus. For example, most strains of Type III pneumococcus are avirulent for the rabbit, although occasionally strains have been isolated which are rabbit virulent. No difference can be shown between the nature or amount of capsular polysaccharide produced by the virulent and avirulent strains, so it must be concluded that factors other than the capsule itself are responsible for the differences in virulence.

It is of considerable interest that although strains of Type III pneumococcus vary greatly in their virulence for rabbits, uniformly they are highly virulent for mice, which indicates that the factors aside from the capsule which are necessary for rabbit and mouse virulence are not necessarily the same. It should be emphasized that virulence can be defined only for the species in which it is tested, and that the findings obtained for one species of animal usually are not applicable to another.

The role of the capsular polysaccharides in virulence of pneumococcus is clear and the same is true for Friedländer's bacillus and *Hemophilus influenzae*. With some other species the function of the capsule is much less clear. Virulent strains of *B. anthracis* are uniformly encapsulated, whereas nonencapsulated strains are avirulent. On the other hand, antibodies directed against the capsule appear to have no protective power whatsoever. Edema fluid from anthrax-infected animals contains an antigen, as shown originally by Bail (1904), which gives rise to active immunity in certain experimental animals. This immunity cannot

be transferred passively. Recently, Grabar and Staub (1946) have presented evidence indicating that the antigen in edema fluid which gives rise to protective antibodies consists of a polysaccharide combined with a protein, and Gladstone (1947) has devised methods for preparing the "protective antigen" in vitro. It is highly significant, however, that avirulent, noncapsulated strains of *B. anthracis* can cause the production both in vivo and in vitro of an antigen which confers active immunity. Following the development of active immunity resulting from injection of the edema fluid antigen or that prepared in vitro by Gladstone, animals can be immunized further by injecting living, virulent anthrax bacilli. The sera from these hyperimmunized animals is capable of passively protecting other animals against anthrax, but absorption of the serum with living bacilli or with antigenic edema fluid (Watson et al., 1946) does not remove the protective antibodies.

These observations have been summarized because they indicate clearly that although a surface component may be uniformly present in virulent, invasive organisms, as in the case of the capsule of *B. anthracis*, other factors may be of equal or perhaps even greater importance for pathogenicity. With the anthrax bacillus, the interaction of the growing organisms and host fluids causes the production of a "protective" antigen. What the role of this antigen may be in the virulence of *B. anthracis* we do not know, but by analogy with "protective" antigens associated with other bacteria, it probably has considerable significance in invasiveness.

We have already seen how staphylococci through the action of coagulase may form a protective surface coating of fibrin derived from the host. Thereafter, other factors, such as toxins formed by the organisms, are able to contribute their part in the production of the lesions. The situation in Group A hemolytic streptococci is com-

parable to that described for staphylococci. Group A streptococci produce a variety of exotoxins: streptolysins O and S, scarlatinal toxin, streptokinase (fibrinolysin) and others. None of these exotoxins can be formed, however, unless the bacteria are able to establish themselves and multiply in the tissues. Two surface components appear to be involved in this process, the M proteins described by Lancefield (1941) and the capsule composed of hyaluronic acid. Type specificity of Group A streptococci depends upon differences in the prolamine-like M proteins, in the same way that the capsular polysaccharides determine type specificity in pneumococci. Their importance in infection is demonstrated by the observation that streptococci from which M protein has been lost are avirulent, and also that type specific antibodies to M protein protect against infection with the homologous strain. Streptococci from which M protein has disappeared may retain their whole exotoxin armamentarium, but yet have lost their virulence.

Most of the studies on the virulence of Group A streptococci have been carried out in mice, and it is by no means clear that the factors involved in mouse virulence are the same as are necessary for human virulence. On isolation from human infections, Group A streptococci are usually avirulent for the mouse, and it is only following repeated passage in mice that the strain becomes virulent. Undoubtedly an extensive process of selection has been involved in this enhancement of mouse virulence because of the many mouse passages that are necessary; accordingly, application to man of findings obtained by study of mouse virulent strains may not be entirely justifiable. One of the changes occurring during enhancement of mouse virulence has been described by Elliott (1945). Certain strains of Group A streptococci produce an extracellular protease of the papain type which digests the M proteins even when present on

the surface of the living streptococci. Such strains are avirulent for the mouse, but enhancement of virulence by repeated mouse passage is associated with a loss in the capacity to produce the protease. The significance of protease production in human virulence of Group A streptococci has not been assessed.

Evidence for the part played by the hyaluronic acid capsule of Group A streptococci in mouse virulence has been obtained by Kass and Seastone (1944) following the demonstration by Hirst (1941) of its importance in the virulence of Group C streptococci for mice and guinea pigs. There is reason to believe that the large capsules composed of hyaluronic acid which are present in young cultures of streptococci inhibit phagocytosis. The studies of Hirst and of Seastone have shown that repeated injections of hyaluronidase, an enzyme that depolymerizes hyaluronic acid, confers some protection on infected mice and guinea pigs. Inasmuch as hyaluronic acid is a normal constituent of tissues and hence not antigenic, evidence for its role in streptococcal virulence cannot be obtained by the immunologic methods so successful in the case of pneumococcus and many other species. This fact, together with the observation of the participation of fibrin in virulence of staphylococci, make it important to use methods other than immunologic ones in studies of virulence.

#### TOXIC SURFACE COMPONENTS AND BACTERIAL VIRULENCE

Gram-negative bacteria, both pathogenic and nonpathogenic, possess a toxic surface complex known as the O, or somatic, antigen. This complex is not present in Gram-positive organisms. The toxic O antigen consists of phospholipid, polysaccharide and protein. In virulent strains the O antigen is uniformly present, and antibodies directed against it confer some degree of protection upon experimental animals. Upon loss of the



O antigen through mutation, the virulence of the strain is lost.

The precise role of the toxic O antigens in virulence of the *Bacteriaceae* is not known, since the relative importance of phagocytosis as opposed to a bactericidal effect dependent upon the presence of specific antibody and complement, has not been defined. Various studies have shown, however, that the O antigens inhibit the bactericidal effect of the antibody-complement system (Thibault, 1939; Cundiff and Morgan, 1941). It has also been observed (Robertson and Yu, 1938) that the O antigens induce leukopenia upon injection and that, moreover, they have an antiphagocytic effect (Morgan and Upham, 1941). Added to these diverse activities of O antigens of the *Bacteriaceae* on the immune mechanisms of the host, are their direct toxic actions. The O antigens are lethal for various experimental animals upon injection of the purified material. In smaller doses they produce a rapid rise in body temperature, elevation of blood sugar and a fall in inorganic blood phosphorus. An additional effect which has been studied especially by Schwartzman (1937) is the production of marked damage to the endothelium of blood vessels, giving rise to increased permeability. It is not clear whether or not all of these pharmacologic effects are due to one and the same component of the toxic O antigen complex. Clarification must await isolation and identification of the toxin itself.

#### THE RELATION OF HYPERSENSITIVITY TO BACTERIAL COMPONENTS TO DISEASE PROCESSES

Although hypersensitivity to components of the bacterial cell undoubtedly plays a considerable part in the type and progress of the lesions in many infectious diseases, especially those of chronic nature, little specific information is available except in the case of tuberculosis. In tuberculosis in guinea pigs, the effect of hypersensitivity to

tuberculo-proteins, which are in themselves nontoxic, can be demonstrated readily. Normal guinea pigs injected with as much as 2 cc. of Koch's old tuberculin do not suffer any serious ill effects. On the other hand, if a tuberculous guinea pig during the eighth or tenth week of infection is injected with as little as 0.01 cc. of tuberculin, death may occur within a few hours. An intense inflammatory reaction occurs at the site of injection and throughout the body wherever tubercles are present.

The generalized tuberculin reaction in the guinea pig which ends in death illustrates the ill effects of the allergic reaction in its most severe form. Less spectacular, but nonetheless damaging effects, due to the hypersensitive state that uniformly exists, and the presence in the lesions of tubercle bacilli and their products, almost certainly occur in the course of the tuberculous process in man and animals. It seems likely that the destructiveness of the tuberculous lesions in adult tuberculosis with extensive caseation and fibrosis may be due in good part to local hypersensitivity reactions in the tuberculous lesions themselves.

In other chronic bacterial infections, for example brucellosis, hypersensitivity to the bacteria and their products likewise may have an important influence on the character and persistence of the lesions. This has not been clearly defined, however.

#### THE COMMUNICABILITY OF BACTERIA

A great deal more is known of the factors influencing the pathogenicity of micro-organisms than of the properties they must possess in order to be communicable from host to host under natural conditions. Pathogenicity is more susceptible to experimental study because of its end result,—disease, whereas organisms may be transmitted from host to host without any detectable pathologic alteration occurring. For this reason, the study of properties con-



cerned in communicability of micro-organisms has lagged far behind knowledge of factors which determine virulence.

Although communicability must be possessed by naturally pathogenic species, it is by no means confined to virulent micro-organisms. The bacteria which comprise the normal flora of the skin, oropharynx, alimentary tract and external genitalia of healthy persons are by definition communicable, even though they rarely cause disease. Indeed, certain of the micro-organisms forming the normal flora of the alimentary canal may be essential for health through the synthesis of vitamins or the partial digestion of nutrients for which the host does not possess enzymes. For example, much of the vitamin K requirement of man and animals appears to be supplied by intestinal bacteria which are able to synthesize it. In ruminants, bacteria in the stomachs play a very important part in the digestion of food.

In contrast to micro-organisms that are communicable but nonvirulent are those that are virulent when introduced into the animal body but are not communicable from experimentally infected animals to normal cage mates. For example, pneumococci are virulent when introduced into mice by almost any route. One or two cocci of the most virulent strains will cause the death of mice following intraperitoneal injection, but normal mice kept in the same cage remain perfectly well.

Since communicability and virulence are not necessarily interdependent, it would appear that epidemic strains of a bacterial species are those in which natural selection has operated to permit the survival of variants which are concurrently highly communicable and virulent.

*The site of the lesions* in the infected host has an important influence on communicability and this may have a bearing on the noncommunicability of pneumococcal infections in mice where the disease does not involve the respiratory tract primarily, even though the bacteria are placed directly

within the trachea, but instead produces a rapidly fatal bacteremic infection. If the disease were localized in the lungs and mice had the capacity to cough and spit, it is possible that pneumococcal infection might occur as an epizootic disease in this species.

The influence of the site of the lesion on communicability is illustrated by plague in man which occurs in two well-recognized clinical forms called "bubonic" and "pneumonic" plague respectively. The bubonic form is spread from rats to man through the bite of the rat flea. Buboes form in areas adjacent to the point where the organisms are introduced through the bite of the flea and though invasion of the blood stream commonly occurs, secondary cases of plague do not arise because the bubonic form is essentially a closed infection. On the other hand, pneumonic plague caused by the same bacterial species, *Pasteurella pestis*, is contagious to those in proximity to the sufferer who expels enormous numbers of plague bacilli during coughing and in the copious discharges from the respiratory tract. Secondary cases of pneumonic plague occur commonly under appropriate environmental circumstances, because the open nature of the pulmonary lesions permits dissemination of the bacilli in enormous numbers.

A less exotic illustration of the influence of the site of the lesions on the communicability of bacteria is afforded by the hemolytic streptococcus. The expression "dangerous carrier" has come into current usage to indicate infected individuals from whom secondary cases arise commonly. Several independent investigations have shown that patients with purulent lesions discharging on the body surface, as for example otitis media, cervical adenitis and wound infections are more likely to transmit infection than patients with uncomplicated streptococcal pharyngitis. The reason for this difference in communicability from different lesions caused by the same streptococcal strains may be because purulent lesions dis-

charge more streptococci into the environment, contaminating heavily everything in contact with the patient, whereas from patients with uncomplicated pharyngitis, fewer organisms are discharged into the environment with consequently less chance of secondary cases arising.

In the case of virulence of bacteria, the *size of the inoculum* is of considerable significance. The same may also be true of communicability, though this has not been demonstrated. When a micro-organism is transmitted from one individual of a species to another of the same species, communicable variants alone are involved, presumably, since only these variants would have survived in the original host. The size of the inoculum here would play only a small part since most of the bacterial population should be communicable variants. In transmission from one animal species to another, however, it is probable that the size of the inoculum is of considerable significance because the conditions necessary for survival in the new host are likely to be different from those in the original host; and if the inoculum is large, there is more chance of communicable mutants being present than if it is small.

In transmission from one host to another of the same species, chance may determine largely whether or not the new host becomes a carrier, although even in individuals of the same species there may be differences which influence the capacity to become carriers. By chance is meant the simple accident of coming in contact with a bacterial species or strain. The operation of chance is well illustrated in the communicability for man of pneumococci (Hodges and MacLeod, 1946). From 40 to 70 per cent of normal adult humans carry one or more of the many serologic types of pneumococci in the pharynx at any given time, some persons carrying as many as five distinct types all at once. The ratios of carriers of two types to one type, three types to two types, etc., as shown in Table 11, are approxi-

mately the same, indicating that, in the main, chance has determined how many types of these communicable organisms are carried by an individual.

TABLE 11. OPERATION OF CHANCE IN PNEUMOCOCCAL CARRIER STATE

Number of carriers of:	
1 pneumococcal type.....	1,317
2 pneumococcal types.....	200
3 pneumococcal types.....	27
4 pneumococcal types.....	4
Ratio of carriers of:	
2 types to carriers of 1 type.....	$\frac{200}{1,317} = 0.152$
3 types to carriers of 2 types.....	$\frac{27}{200} = 0.135$
4 types to carriers of 3 types.....	$\frac{4}{27} = 0.148$

Further analysis of pneumococcal carriers, however, shows that host factors also operate in determining whether an individual will become a carrier of a particular pneumococcal type (MacLeod, Hodges, Heidelberger and Bernhard, 1945). In a population of which half the members were immunized against pneumococcus Types 1, 2, 5 and 7, it was found that significantly fewer of the immunized men were carriers of these types as compared with the non-immunes. It is apparent, therefore, that pneumococci are not as communicable to immune persons as to nonimmunes. Analogous data are not available for other bacteria, though a similar state of affairs probably exists for diphtheria bacilli also. In this case, immunization of approximately half of the susceptible population affords a very considerable measure of protection to the nonimmunized portion, as was found true also for pneumococcal infection in a partially immunized population. The chain of transmission from one susceptible individual to another has apparently been broken by the interposition of immune individuals, who are less able to carry the



organisms. In other words, the immune status of the individual, in the case of pneumococci and probably diphtheria bacilli, exerts a powerful influence on communicability.

Although with certain bacteria the immune individual is less likely to become or remain a carrier than the nonimmune, the *capacity of many bacterial species to survive in immune subjects* is an important contributing factor in communicability. An obvious example is the persistence of typhoid bacilli in the biliary tracts of a proportion of those who have recovered from the disease, whence the organisms are discharged into the intestinal canal and through subsequent fecal contamination of food or water become transmitted to susceptible individuals. If the reservoir of infectious agents consisted only of persons actually sick with infection, the control of infectious disease, by quarantine of the sick, for example, would be a relatively simple procedure. However, since perfectly well individuals, either recovered from infection or who at no time have shown clinical evidence of disease, may harbor fully virulent pathogens, it is apparent that for most infectious diseases quarantine of the sick can never be an effective control procedure. From the point of view of the parasite, the capacity to exist in a healthy person is an important survival mechanism, because if only the actively sick were carriers, the bacterial species would be quickly wiped out whether the infected individual died or survived. The persistence of virulent pathogenic bacteria in persons recovered from infection is a very common phenomenon and occurs with many bacterial species such as hemolytic streptococci, typhoid bacilli and dysentery bacilli.

The development of a carrier state in normal individuals, who at no time have shown evidence of disease, is of more significance in some instances than the persistence of the agent in recovered patients. This is clearly true in the case of meningococci,

where the number of normal persons who carry the micro-organism exceeds by far those who develop meningococcal infection. In this instance, the person who has never been ill is of greater importance in the transmission of the micro-organism than those recovered from infection. With the typhoid bacillus, on the other hand, the individual who has recovered from the disease is the primary source for the maintenance and dissemination of the micro-organism. It is apparent, therefore, that no general rule can be laid down concerning the circumstances under which pathogenic organisms can best survive in the body. With different micro-organisms, affecting different areas of the body, the conditions of parasitism vary.

*The ability to survive outside of the animal body* in which disease is produced or in which they can be intermittently carried may have considerable significance in the communicability of many bacteria. The most striking illustrations of organisms having the ability to survive outside the animal body are the spore-forming pathogens. Anthrax spores may survive in pasture land for as long as 12 years as shown by Pasteur (1881), and animals feeding on it may be infected. The spores of the tetanus bacillus and the anaerobic bacteria associated with gas gangrene, which find their way to the soil especially through the feces of man and animals, survive there for long periods of time, and upon introduction into wounds, may germinate and cause disease. In the case of organisms existing only in a vegetative phase, the capacity to survive outside of the animal body is not nearly so great as for the spore formers.

Numerous studies of the environment have been made in attempting to explain the epidemiology of hemolytic streptococcal infection of the respiratory tract. It has been shown that Group A hemolytic streptococci can survive in floor dust and may also persist on articles of clothing or bedding. Similarly, pneumococci can be isolated from dust both in the environs of per-



sons sick with the disease, as well as from dust of common meeting places.

With air-borne pathogens relative humidity is probably of considerable importance, a dry atmosphere favoring greatly both survival and dissemination. Bacteria, if dried rapidly and maintained in the dry state, survive indefinitely, whereas under conditions of slow and incomplete dehydration, that is, where relative humidity is high, the capacity of bacteria to survive is much less, and, in addition, they are fairly quickly sedimented from the air and not so readily redispersed as in environments of low relative humidity. The influence of relative humidity may perhaps explain the higher incidence of streptococcal diseases, including rheumatic fever, in certain of the Rocky Mountain states than elsewhere in the United States except in the most crowded urban areas of the Eastern seaboard. The low relative humidity prevailing in the Rocky Mountain area is probably conducive to survival of respiratory tract pathogens expelled into the air, and when this is coupled with low environmental temperatures during the winter months, with consequent further desiccation of room air by heating appliances, a better opportunity is given to the organisms both to survive and to be disseminated.

Except for respiratory-tract pathogens, the ability to survive in dust and air is probably of little significance. Survival in water has an important bearing on communicability of enteric pathogens such as those causing typhoid or paratyphoid fevers, dysentery and cholera, all of which may be water-borne diseases though not transmitted in this manner exclusively.

*The ability to survive and multiply in an intermediate host or vector* is of prime significance with some micro-organisms, of which plague is a good example. The plague bacillus causes natural disease in many species of rodents in different parts of the world and is transmitted to man through the bite of various species of fleas which

are ectoparasites of the rodents. The bacilli are able to multiply and cause disease in the upper portion of the alimentary tract of the fleas, which derive their infection from the rodent reservoir. The ability of plague bacilli to infect fleas is thus a crucial factor in their communicability. *Pasteurella tularensis*, an organism related to the plague bacillus and the cause of tularemia, has its reservoir also in various rodent species, and may be carried by arthropods such as ticks and deer flies, which feed on the rodents. Although the disease in man is most often acquired during the skinning and dressing of wild rabbits, it is also possible for transmission to occur through the bite of the arthropod vectors.

Flies (*Musca domestica*) have long been incriminated in the transmission of typhoid fever. In this instance, however, the insect occupies a relatively passive role in that the fly is not infected by the typhoid bacillus, nor does it become a permanent carrier of the micro-organisms. The feet of the fly and other parts of its body surface become contaminated during contact with feces, and the organisms may then be mechanically transferred to human food.

From this discussion it can be seen that certain of the general properties necessary for communicability of various bacterial species can be defined. However, as noted earlier, little information of a specific nature is available. With pathogenic species, the communicable organisms carried in the body usually possess the cell structures known to be associated with virulence though this is not uniformly true. Pneumococci isolated from normal humans are in the encapsulated, that is, potentially pathogenic state, and the nonencapsulated, rough variants are not found. On the other hand, hemolytic streptococci isolated from carriers not infrequently have lost the M protein, which is known to be concerned in their virulence. These organisms must therefore possess a component or components,

other than the important M protein, which prevent their destruction by the body. It can be argued that such avirulent variants, lacking M protein, may have no pathogenic

significance. However, upon transmission to a new host it is entirely possible that they may regain the capacity to produce M protein and become fully virulent once more.

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5

## Response of the Host to the Parasite

In the section on parasitism and disease the general biologic significance of the reactions of host and parasite was introduced. Disease-producing organisms are not accepted without protest. And while the establishment of infection appears to be dominated by the capabilities of the organism to override the influences encountered in its immediate environment, the active responses of disease and resistance represent attempts of the host to assume the aggressive. An appreciation of the responses of the host requires a view of the mechanisms which interpose barriers to the progress of the invader from the time of its arrival until the conflict is decided or an armistice is reached.

### NATURAL MECHANISMS OF RESISTANCE

#### PHYSIOLOGIC BARRIERS AT THE PORTAL OF ENTRY

The first line of defense encountered by a bacterium is furnished by the physiologic conditions obtaining on the body surface or in the cavities to which the agent gains entrance. The character of the tissue and its cell type, the nature of the local secretions and excretions, the drainage, the acidity, are all factors which may play determining rôles in the capacity of the invading organism to survive. An organism may

vary in its behavior as a pathogen, therefore, according to the site where it is deposited.

*The nose and nasopharynx* constitute the major portal to the respiratory tract. It has been said that the majority of organisms are stopped in the anterior nares, where numerous hairs undoubtedly filter out many particles. The respiratory epithelium of the nose is furnished with cilia and a heavy coating of mucus. These agencies serve with high efficiency to entrap bacterial material and then to propel it toward the pharynx. But the fact that the nasal secretions of normal individuals are sterile or contain a limited number of organisms, most frequently staphylococci, is evidence that bacteria are probably killed or rapidly prepared for disposal in that area. Among the known agencies is that of lysozyme, described by Fleming, an enzyme in the nasal secretions and tears which causes dissolution of a variety of nonpathogenic bacteria. Intimations of other inhibitory enzymes have been reported. Moreover, the nasal secretions possess antibodies probably derived from blood serum and interstitial fluids which exude in low concentrations into the nasal cavity. They have been shown to increase after infection with influenza virus and to be enhanced by vaccination. The activity of immune substances at the site of localization is also suggested by the fact that the antibody level of the blood is

a measure of the ability to prevent diphtheria in the pharynx. In addition to these fluid agencies a variety of cells is ordinarily found in secretions; they increase with irritation and may well function in the disposal of particulate matter. The lymphatics draining the nose and nasopharynx are a diffuse, superficial plexus through which many organisms may be drained to the regional lymph node filters and disposed of. Virulent organisms may undergo further multiplication and escape into the blood stream. Although the manner by which the penetration of the mucous membrane takes place is not well established physiologically, studies of the absorption of lead after intranasal instillation of lead carbonate suggest the probability that the material was transported through the membrane by cells (Blumgart, 1924). On the other hand, some bacteria and dyes actually penetrate the olfactory portion of the mucosa by an intercellular route (Rake, 1937).

The significance of a normal mucosa in maintaining the protective mechanism is shown in the tendency to chronic infections by some of the mucoid saprophytes when it is altered. In atrophic rhinitis *B. mucosus ozenae* becomes a frequent resident and *B. rhinoscleromatis* grows freely on a hypertrophic mucosa. The alteration of respiratory epithelium in vitamin A deficiency is commonly associated with infections due to parasites which are not prominently pathogenic. It seems probable that the establishment of more pathogenic organisms may be related to vasomotor changes occurring under the influence of heat or chilling; certainly the influence of dust is seen in the increased incidence of respiratory disease among those working in the dusty trades. It is probable that the basic agencies are overwhelmed by inanimate particles so as to retard clearing, and entrance of the living organisms is thereby promoted.

The majority of agents infecting the respiratory tract are essentially established on the surface. Pneumococci, diphtheria bacilli,

meningococci, streptococci tend to create a focus of multiplication on the membranes from where they can extend. The primary site of localization of a number of viruses appears to be on the superficial epithelium of the respiratory tract. Influenza virus clearly attacks the ciliated respiratory epithelium; the viruses of chickenpox, smallpox and measles probably have their early development in the nasopharynx. And it must be remembered that some degree of inflammatory reaction, even though mild, occurs in response to most injurious agents, thus rapidly modifying the conditions of penetration, drainage and disposal. It is also of interest to point out that when bacteria, such as those mentioned above, produce infection of the lower respiratory tract, they usually are established as well in the upper respiratory tract. For this reason, posterior nasal or nasopharyngeal cultures ordinarily give better information of lower respiratory conditions than those taken from the intermediate oropharynx which has a different, squamous epithelium.

It is probable that mechanisms similar to those encountered in the upper respiratory tract are at play in the trachea and bronchi. Nevertheless, Drinker and Yoffey (1941) state that drainage from the lymphatics of the area is ordinarily slight and immune substances are less readily transmitted through these membranes from the blood, although antibodies to influenza virus are demonstrable in sputum. While cilia of the epithelium furnish an ingenious mechanism for the removal of particulate matter, viscous oil or the heavy mucus of the common cold can descend against the action of cilia, taking with it organisms which may be protected, and find lodgment in the lungs to initiate pneumonia. It seems likely that other factors which are considered predisposing to pneumonia act through an inhibitory effect upon local clearing agencies. Drunkenness or exhaustion leading to heavy sleep, exposure to cold, dusty work, all tend to reduce the efficiency of the normal local



processes of removal, and all predispose to acute infection of the lower respiratory tract.

Entrance of bacteria into the lung directly through inspired air has been little studied. Nevertheless, experimental studies of organisms in droplet nuclei sprayed into the atmosphere clearly point out that infectious agents can reach terminal bronchioles widely. On the other hand, it has been repeatedly shown that fluid material more readily flows into the lungs than dry particles do. The alveoli are richly supplied with lymphatics, and there are numerous scavenger cells which seek to remove organisms through regional lymph nodes. Moreover, they are completely surrounded by the capillary blood stream which may readily furnish phagocytes and antibody serving to extinguish a variety of organisms. Under normal conditions a significant number of organisms does not persist in the alveoli, but tissue injury or irritation alters the balance.

Organisms which evade the influence of these mechanisms may become established in the respiratory tract as part of the normal flora. They are either naturally fitted to the conditions encountered or become adjusted to it, but to attain the latter state the ability of the organism to induce injury or inflammatory response must be limited. The number of species ordinarily found in the respiratory tract is small, and it is probable that only under abnormal conditions do actively pathogenic organisms persist. In this respect the participation of bacterial antagonism, the capacity of one or a series of organisms established in the area to inhibit the colonization and growth of other organisms, may well be an important adjunct to the intrinsic responses of the host.

*The alimentary tract* harbors a much wider variety of organisms than is found in the respiratory tract. The different areas, the mouth, oropharynx, stomach, small and large intestines, tend to have their charac-

teristic inhabitants. This of itself illustrates the effect of local conditions. The *mouth* presents a squamous mucous membrane, the usual lymphatic drainage, a characteristic pH, lysozyme, saliva and its additional activities, and numerous areas in which traumatic injury repeatedly occurs. The classic studies of Bloomfield (1922) clearly show that particles entering the mouth are readily swept toward the base of the tongue and swallowed. But the influence of most of the other agencies is little studied. The manner in which organisms are destroyed in the mouth clearly points to other than mechanical effects, and the idea is supported by the fact that saliva apparently has an inhibitory action upon diphtheria bacilli, upon numerous cocci and organisms pathogenic for the intestinal tract. There is also reason to believe that in this area, too, exuding components of blood serum participate in disposal.

The *throat* or oropharynx is an area of considerable importance since it serves both the respiratory and alimentary tracts. The flora commonly differs, however, from that of the mouth and approaches more that of the respiratory tract. It certainly is a zone of rapid drainage and frequent localization of harmful agents. The tonsils, whose function is not certain, are especially subject to injury and may become chronically infected with pathogenic hemolytic streptococci or diphtheria bacilli, creating dangerous carriers of infection.

The highly acid gastric juice of the *stomach* tends to destroy a great number of organisms. Nevertheless, the fact that *Salmonella* and *Shigella* infections or cholera can be acquired by ingestion of the organisms indicates that it is not a complete barrier to organisms in the buffered medium of food. Under normal conditions, however, it tends to maintain a sterile environment, although acid-fast tubercle bacilli can survive. Diseases such as pernicious anemia or gastric malignancy in which gastric acidity is reduced, are usually associ-



ated with the growth of otherwise foreign organisms in the stomach.

In the stomach and particularly in the colon, the presence of mucus and lysozyme appears to participate in the protective action. The variations in acidity, in character of secretions, in anatomic structure and in the nature of their contents tend to determine the composition of the populations of the different areas. Diet of itself can be an important influence. The fact that milk and carbohydrate will enhance the growth of lactobacilli and *Escherichiae* and that reduction in these articles of diet will limit their growth, while a high meat diet appears to increase the growth of the proteolytic Gram-positive organisms, may be indicative of environmental changes which could be advantageous to the host.

The *vagina* behaves differently at different ages. In the newborn period, under the hormonal influence of the mother, the reaction is strongly acid, probably through the production of lactic acid from glycogen by tissue enzymes but more through the action of the highly acidic Döderlein's bacilli. Shortly thereafter, Döderlein's bacilli disappear. Glycogen is not found, and the alkaline environment is then occupied by mixed organisms, and susceptible to gonococci. With puberty, glycogen, Döderlein's bacilli, lactic acid and an acid pH reappear under the influence of estrogen, and a change in the character of the epithelium is observed. While other organisms may survive, this environment no longer supports gonococci, and artificial modification of the environment by estrogen may be used for treatment of gonococcal vaginitis in the child.

The outer surface, the *skin*, also has the capacity to destroy numerous bacteria which alight. Few agents penetrate the intact integument, but wounds and tissue injury furnish a medium in which organisms can survive and multiply. Staphylococci or streptococci can become established in the hair follicles and there establish destructive le-

sions. The sterilizing mechanisms are not well defined; the sebaceous secretions and perspiration seem certainly to participate. It has been suggested that sweat acts through its acidity and salt content; other chemical constituents of the skin may be involved. Lysozyme is also present. Numerous influences of sunlight and vitamin D have been suggested, but they are not well understood.

It is evident that numerous factors which serve to maintain a normal physiologic state in the orifices and on the surface of the body fulfill a function in limiting the opportunity for microbiologic agents to establish "beach-heads" from which they might extend their operations.

#### GENERAL RESPONSE TO NONPATHOGENIC ORGANISMS

Beyond the local environmental conditions at the various portals of entry which serve defensive functions in the removal of the less virulent and less-adjusted organisms, there are available against infection processes of protection of a general nature which constitute a broad physiologic function directed toward the disposal of substances foreign to the body. It is generally believed that these mechanisms of the normal host were basically associated in the evolutionary sense with the ability to take in and digest foreign material for nutritional purposes. Metchnikoff pointed out that lower zoologic species, such as the amoeba, obtain food largely through the actual ingestion of particulate matter by the animal cell which then digests the assimilable part and extrudes the residue. In the higher animals the capacity to take up particulate matter is more sharply the function of certain definite cell types. The phenomenon of ingestion of foreign particles by the cell Metchnikoff termed phagocytosis and the cells, phagocytes. The large mononuclear cells were termed macro-

phages and the polymorphonuclear leukocytes, microphages.

#### PHAGOCYTOSIS OF NONPATHOGENIC AGENTS

The mode of action of the system is readily demonstrated by studies of the distribution of the carbon particles of India ink after intravenous injection. Within a few minutes the particles are found in large numbers of the macrophages or fixed histiocytes forming the endothelium of the sinuses of the liver (the Kupfer cells), of the spleen, of the bone marrow; and the monocytes and polymorphonuclear cells of the blood rapidly take up the dye (Cappell, 1929). The latter cells collect in the lungs as if filtered out or selectively concentrated in that area. There are other collections of extremely active cells which particles of this size do not reach after intravenous injection because they do not pass the capillary walls; when given by other routes the material rapidly accumulates in the histiocytes of the regional lymph nodes which constitute major drainage depots. In addition, the wandering tissue macrophages, numerous in practically all connective tissues, readily phagocytize the dye. The material may be stored in them for considerable periods of time until the cells themselves deteriorate and become subject to removal through the lymphatic system. These macrophages or histiocytes most active in taking up particles of dye were called by Aschoff the reticulo-endothelial system. One may look upon it as an organ whose function is to remove foreign material, perhaps still of basic importance in the assimilation of certain types of food stuffs not readily digested by first intention.

It is extremely interesting that nonpathogenic bacteria are handled in much the same way as carbon particles. Organisms given intravenously are promptly taken out in the endothelial cells of the liver, spleen and bone marrow, but, in addition, the polymorphonuclear cells accumulate in great

numbers in the lung and ingest them. Numerous investigators have noted the chain of events following the injection of different organisms in various species of animals. The sequence is so similar under a wide variety of conditions as to make it apparent that this process constitutes a basic physiologic clearing mechanism. Within two or three minutes ingestion is well under way, and the blood is largely free of the organisms in 30 to 60 minutes. The bacteria are digested in the cells and disappear. Moreover, when the inoculum is heavy many of the polymorphonuclear cells which have engulfed organisms are transported to the liver and spleen where they and their cargo are finally disposed of. As the virulence of the test organism is increased it becomes apparent that a certain number of the bacteria escape destruction even though they are filtered out in the organs. They remain extracellular or may escape from cells and after a period begin to multiply and reappear in larger numbers in the blood stream, eventually leading to overwhelming generalized infection. The interval between the time of injection and that at which free multiplication of the organism begins was called by Bull (1916) and by Bartlett and Ozaki (1916) the period of adaptation, and Bull suggested a relationship between this interval and the incubation period of disease. Phagocytosis then represents a major feature of the host's effort to dispose of both animate and inanimate particles of extrinsic nature.

#### NATURAL ANTIBODIES

In the pursuit of such studies it was noted in microscopic sections that aggregation of organisms had frequently taken place before they were ingested by the cells, and Nuttall, as early as 1888, had observed that defibrinated blood appeared to kill certain organisms. Then in 1903 Wright and Douglas demonstrated that normal unheated serum commonly possessed the capacity to



act upon a number of bacterial species to prepare them for phagocytosis by normal leukocytes; little ingestion took place in the absence of serum. The effective substances in serum were named *opsonins*. Moreover, it was demonstrated that heating the serum at 56° C. largely destroyed the opsonic effect which could again be restored by the addition of normal fresh serum. The thermolabile fraction was called "alexin" by Bordet, and "complement" by Ehrlich, the latter name indicating the capacity to complement the activity of the thermostable portion of the complex. Phagocytosis is, therefore, not simply a cellular activity but one in which components of the body fluids participate, a joint cellular and humoral function. Normal blood sera also possess the power in the presence of complement to cause lysis, disintegration, of some species of bacteria or of erythrocytes from other species of animals.

The serum components which bring about phagocytosis, agglutination or lysis of a given bacterium by sera of individuals who have not had previous experience with that organism in specific infection are referred to as natural, normal, innate, physiologic or nonspecific antibodies. They represent serum globulins which have been oriented in some manner to combine with components of the organism and render it susceptible to destruction, a form of native physiologic intelligence.

The manner in which orientation of the globulin to react with various organisms arises is far from settled. One view is that of chemical accident. A complex chemical structure such as a bacterium contains substances which may be chemically related to organisms of different genera and species. For example, the capsular polysaccharide of the Type II pneumococcus is similar to that of Type B Friedländer's bacillus and to others of plant origin. The Vi antigen of *S. typhosa* is found in high concentration in certain strains of *E. coli*. Numerous animal tissues contain lipopolysaccharide sub-

stances, Forssman antigen, which upon injection into other animals induce formation of antibodies to sheep erythrocytes. Many instances of such chemical cross relationships exist. It is not unlikely then that in some cases the natural antibodies acting with certain bacteria are incidental to experience with another organism or with other foreign substances containing chemically related compounds which have induced alterations in the globulin to aid in their detoxification or in their assimilation.

Still another thesis attributes development of these characteristics to a genetic experience in which the development of the reactive globulins is determined by an evolutionary pattern in the same manner as that of the iso-agglutinins which establish the blood groups of a species or result in hemagglutinins against erythrocytes of other species. The specificity of the antibody toward bacteria may, nevertheless, still be incidental. There is good evidence to support the concept of genetic differences in the capacity of individuals and of species to respond to certain antigens, that is, in their immunizability, and a developmental influence is observed in the fact that young animals respond, in general, less efficiently than adults. This is probably related, however, to the genetics of globulin formation rather than to a hereditary schedule which governs the response to each of a number of individual agents. The inherent ability of serum globulins to react with certain bacteria may, in an evolutionary sense, be in keeping with the capacity of the infant animal to digest certain food stuffs from the start.

A third hypothesis as to the origin of natural antibodies is that they develop as a result of experience with the specific agent in unrecognized or subclinical infection. But antibody derived under such conditions does not represent natural antibody in the sense heretofore employed, for immunologically those individuals are no longer normal nor is their serologic beha-



avior native or innate. Continued ingestion and digestion of nonpathogenic organisms might well enhance the production of globulin to aid in their disposal or in the disposal of chemically related substances. Ordinarily, however, this does not reflect the character of the encountered agent to the same degree as is the case after actual infection and invasion by pathogens. The adult rabbit usually possesses opsonins for Type II pneumococci, but it is improbable that they develop as a result of uniform infection of the species by those organisms. Nor is the bactericidal action of normal chicken serum against those organisms derived from infection. On the other hand, the human individual with his numerous exposures might well be influenced by incidental experience with bacterial analogs or by the effect of repeated mild infections with the organism itself. Consequently, the behavior of the serum of "normal" human adults toward commonly encountered pathogens cannot properly be compared with that of animals not ordinarily exposed to infection by those agents. The same criticism may apply to studies of reactions to enteric organisms in the serum of animals constantly exposed to excreta and garbage, as compared with man.

The natural antibacterial antibodies are not highly specific; they do not withstand storage; they are not readily capable of being transferred to other animals for protection; for phagocytosis they require fresh serum and they can be absorbed by substances or organisms not specifically related. Kelley (1932) demonstrated that the protective action of hog serum against Type II pneumococci could be absorbed by treating the serum with avirulent organisms and not by the type-specific polysaccharide. Ordinarily they are absorbed from serum with greater difficulty than are specific antibodies. In many respects they appear to be directed more against the organism as a whole than against specific antigens. It may be that the broad type of resistance elicited

by repeated inoculations of nonpathogenic organisms such as rough pneumococci (Tillett, 1928) is induced by the enhancement of the same globulin reactants as are operative in the natural or nonspecific states. And the constant experience of removal and digestion of avirulent organisms may result in a greater potentiation or augmentation of the globulins and cells involved.

The importance of these natural antibodies in immunity is not well established. A number of observations in different species of animals has suggested a correlation between their presence and the species' immunity, but in other instances they are found in the serum of animals susceptible to the particular organism. Moreover, the phagocytic action for virulent pneumococci may exist in the blood of patients at the time they take sick with pneumonia due to the same organism (Robertson, et al., 1930). The same phenomenon has been demonstrated in dogs undergoing experimental pneumonia (Terrell, 1930). In fatal cases the capacity rapidly disappeared. It is of interest that in bloods from the general population the highest frequency of phagocytosis was observed against Type II pneumococcus, the polysaccharide of which has many relatives in nature (Sutliff and Finland, 1932). The behavior of the natural phagocytic mechanism in general is such as to indicate that it can play a role in limiting infection, perhaps through reducing bacteremia, especially with respect to organisms of low or moderate virulence.

In addition to the mechanisms which act directly upon bacteria, there are those which combine with and neutralize soluble exotoxins of bacterial origin. These substances are poisons which exude from the site of injury. It is difficult to conceive of their fixation by normal cells as a defensive mechanism on the part of the host rather than as a selective action of the toxin. Diphtheria, scarlet fever and tetanus have been the three conditions which have furnished most information as to the distribu-

tion of antitoxin in general populations. Here again, the immunologic use of the word "normal" is difficult. In agreement with the thesis of Hirzfeld there appear to be examples of genetic influences participating in the development of antitoxins under "natural" conditions. In contrast to the natural antibacterial antibodies, however, the bulk of evidence points to the probability that the acquisition of antitoxins to diphtheria or scarlatina are primarily related to infection with the respective organisms. Much of the disagreement as to the origin of natural antitoxin seems to reside in the question as to whether infection is recognized as disease and in the meaning of the word "normal." The age curve of development of diphtheria antitoxin has generally been found to conform to the prevalence of the organism and of the infection. There is little doubt that the prevalence of unrecognized infection by *C. diphtheriae* is far greater than that of clinical diphtheria. In animals such as horses, and in tropical areas, the frequency of cutaneous diphtheria may be an important item in the building of antitoxin. Another reason that the relation between specific infection and the presence of antitoxin appears so clear is that these toxins have few chemical relatives in other organisms which could furnish antibody by indirect or non-specific stimuli. The demonstrable "normal" antitoxins are heat-stable, and their presence in the serum of man is intimately associated with resistance to the respective diseases. The antitoxin unites directly with toxin to neutralize it without requiring the intermediary of cells or complement. Nevertheless, some species of animals, such as the rat, are essentially refractory to diphtheria toxin although antitoxin is not demonstrable in their blood, indicating that their cells are not susceptible to the action of toxin—an entirely different biochemical mechanism of natural immunity from those previously discussed.

## GENERAL RESPONSE TO PATHOGENS

The pathogenic organism is one which arrives with the capacity to survive in a host and whose multiplication results in injury which, in turn, elicits an active response from that host. But the primary reaction of the host is to mobilize the same natural mechanisms of globulins and cells which effect the clearing of inert particles and nonpathogenic agents. The local physiologic processes and the normal clearing mechanisms come into action and organisms which enter the blood are rapidly removed. Some of them, however, are not destroyed and begin to multiply. The subsequent events in most diseases of man have not been well observed; however, through experimental infection, occasional early necropsies, and study of local conditions certain features have been reproduced and a common sequence reconstructed. The period between the decision that the organism will survive the immediate influences so as to institute infection and the onset of the recognizable clinical disturbance which constitutes disease is the *incubation period*—an interval of scientific darkness so far as the behavior of the competing biologic systems is concerned.

With the beginning of growth the products of the pathogen cause injury to the local tissues, arousing an inflammatory response which seeks to localize the injury. Edema fluid accumulates from the dilated capillaries and fibrinogen exudes into the area creating a fibrin network and thrombi which occlude the enlarged lymphatics so as to limit the dissemination of organisms from the focus. This effort at fixation is accompanied by the accumulation of polymorphonuclear leukocytes from the blood stream and phagocytosis begins. The attraction of phagocytes to the bacterial cell has been termed positive chemotaxis, as opposed to a repellent or negative chemotaxis, and appears to be a strong influence. Menkin (1940) has described the preparation



from inflammatory exudates of a "nitrogenous substance" called leukotaxine which apparently induces increased capillary permeability and the rapid migration of polymorphonuclear cells into the area. As will be seen, the amount of phagocytosis by these cells at this stage of the infectious process varies with location of the lesion, with the organism and with the animal.

Even the fever associated with pathogenic injury appears to have an influence beneficial to the host. Phagocytosis may be increased, and some organisms may actually be prevented from development at the levels of temperature reached by the infected body. There are repeated observations of the inhibition of virus infections in small animals kept at high environmental temperatures.

#### PHAGOCYTOSIS OF PATHOGENS

Polymorphonuclear cells endeavor to fulfill their first-line function of engulfing and seeking to dispose of the pathogen, but their efficiency is decidedly less than with nonpathogenic organisms. Phagocytosis may be slight in amount, many of the cells are injured and disintegrate; in other instances the bacterium is engulfed and transported to the clearing depots, but the polymorphonuclear cells are unable to digest them and may, under these circumstances, actually serve to distribute infection. As the leukocyte wave deteriorates, the mononuclear macrophages begin to make their appearance and increase remarkably in number. Menkin, in studying this transition, has called attention to the fact that with the appearance of macrophages the acidity of the inflammatory process increases, and he considers this change as the determining factor in the immigration of monocytes. These cells seem to come both by transport from the blood and, in many instances, by migration from the local tissues. There are reasons to believe, moreover, that the destruction of polymorphonuclear cells may

of itself release a substance which calls forth the macrophage. In some infections the polymorphonuclear phase is brief and the macrophage is rapidly mobilized. But this pattern of primary polymorphonuclear excursion succeeded by the monocytic is the common response to a variety of organisms as different as the pyogenic cocci, typhoid bacilli and tubercle bacilli. Nevertheless, the speed of development of the various stages is not constant, and the character of the tissue involved strongly influences the pattern of invasion. Early in the process of inflammation, as the organisms multiply, they may gain entrance to the lymph channels, reaching the regional lymph nodes and from there progress to the blood stream where the general clearing mechanism comes into play. In other instances, as the blood capillaries are distended their permeability increases, and entrance of organisms to the blood may be gained in this manner. Again, direct tissue injury caused by the bacteria may permit the penetration. In most instances the actual presence of organisms in the blood stream represents overflow from the tissue and is called *bacteremia*. In some cases, however, as in staphylococcal infection, the bacteria may multiply and be transported elsewhere where they initiate new foci of infection. The more virulent organisms tend to invade the tissues rapidly and in this their chemical or antigenic constitution appears to play a determining rôle. It has been repeatedly demonstrated that the capsular polysaccharide of the pneumococcus inhibits phagocytosis and that fully virulent pneumococci are, in most species of animals at least, quite resistant in the early stages of infection to phagocytosis by polymorphonuclear cells. Although a specific close relationship between a single chemical structure and the capacity of the organism to escape ingestion is not uniformly established, the probabilities are that similar capacities are possessed by many organisms. This capacity was traced by Bail to



aggressins, indicating a mechanism of offensive action in the micro-organism.

#### INFLUENCE OF PATHOGENIC MECHANISMS

Pneumococcal infection of the lung is one of the most striking examples of classic inflammatory reaction to infection at the surface of a tissue. One may find all stages of response in a single infected lobe; at the advancing margin there is edema fluid in the alveoli, hyperemia and swollen lymphatic vessels in the walls. Next is a zone in which pneumococci are growing freely in the fluid just ahead of the polymorphonuclear cells which are accumulating rapidly even though they are incapable of destroying the encapsulated virulent organisms. Fibrin is then laid down, and in this area the number of bacterial and polymorphonuclear cells tends to be greatly reduced, indicating that some process of bacterial inhibition is operating. In these latter two areas, efforts toward phagocytosis by the polymorphonuclear cells are continued even though the organisms are not destroyed. In the oldest part of the lesion, resolution of the infectious exudate may be taking place with large mononuclear phagocytes rapidly digesting and removing the debris.

The staphylococcus, producing a characteristic infection in the skin, exerts a very rapid action. Menkin has pointed out that staphylococci, which tend to cause abscess formation, have a high capacity to cause necrosis of tissue, to incite the formation of thrombi in the lymphatics and the so-called fixation of the infectious process in about one hour. Diffusible dyes introduced into the area are held in place, as opposed to their ready dissemination through regional lymphatics in the normal animal. With pneumococcus, Type I, about 17 hours are required to reach a similar stage of fixation after cutaneous infection; whereas with beta hemolytic streptococci, 45 hours or more elapse before the localizing process is advanced to the stage where passage of dye

from the infected area is impeded. In this respect it is interesting to note that staphylococci possess a potent coagulase which can cause prompt clotting in the blood vessels in addition to the direct tissue destruction caused by their other enzymatic agencies. The beta hemolytic streptococci, in contrast, possess an enzyme which dissolves fibrin, and it seems not unlikely that this factor accounts for the tendency of these organisms to spread quickly through the lymphatics and to extend readily along tissue planes with rapid invasion of the blood stream. These three species of organisms, in their stages of invasion and rapid growth, multiply and exist as extracellular organisms, producing disease by the products of rapid growth and the intensity of the response they elicit. Tubercle bacilli, however, are readily ingested by the polymorphonuclear cells, but the organisms are resistant to digestion by that type of cell, and soon the remaining free bacteria, as well as those in the leucocytes, are found in the accumulating mononuclear phagocytes. Consequently, the majority of tubercle bacilli take up an intracellular residence in monocyctic cells which serve to maintain them and to aid in their transportation. The mode of progress of the typhoid bacillus has not been well established. It apparently does not multiply readily within the gastro-intestinal tract or in the blood stream. On the basis of histologic studies early in the course of typhoid fever Goodpasture (1937) has suggested that a toxic component of typhoid bacilli organisms causes destruction of macrophages and that they then assume an intracellular state in collections of plasma cells in lymphoid tissue, at least during the incubation period. His studies, and those of his colleagues, in chick embryos have pointed out that *Str. viridans*, *A. aerogenes*, *Br. abortus*, *M. tuberculosis* and *S. typhosa* all readily invade cells in or about the lesions and utilize this environment for their maintenance. *C. diphtheriae*, like staphylococci, streptococci and

meningococci, showed no evidence of intracellular localization, although injury to the blood vessels takes place promptly, and, in some instances, focal necrosis of the membranes was observed (Goodpasture and Anderson, 1937; Buddingh and Womack, 1941).

It is clear, then, that while the fundamental function of phagocytosis is carried out against nonpathogenic organisms, the virulent bacterium escapes or may actually use the phagocyte for growth and maintenance. Ordinarily, extracellular organisms are thought of as being in a state of rapid multiplication because of little defense from the host. When meningococci in the spinal fluid are extracellular the disease is progressing rapidly; when they become largely intracellular, resistance is in effect. Similar circumstances apply to the gonococcus: its persistence in an intracellular state is commonly evidence that the infection has become stabilized. A generalization might be made that organisms which seek intracellular adaptation are characteristically related to diseases with prolonged chronic courses. Although from the point of view of the parasite it has found a suitable habitat, the assumption that this is an entirely one-sided arrangement is not justified, for, by retaining the organisms within the cell, the degree of invasion is limited, and the severity of the disease is undoubtedly reduced as opposed to florid infection in which uninhibited growth in the body fluids take place.

The cellular behavior has become a dominant feature of the inflammatory response to organisms, but there are scattered observations suggesting that the natural antibody output may also be increased. Moreover, the early appearance in disease of a serum fraction capable of flocculating pneumococcus "C" is evidence of humoral responses (Tillett and Francis, 1930). The serum of mice infected with poliomyelitis virus may possess a neutralizing capacity for that virus at the onset of paralysis in

the early days of the disease (Brown and Francis, 1945). Other evidences of the accumulation of an inhibitory factor in serum in acute illness can be found, but this entire field needs further exploration. In systemic disease it might be expected that features of the general effort to limit invasion would be reflected in the serum.

## EMERGENCY MECHANISMS OF RESISTANCE

### DEVELOPMENT OF SPECIFIC ANTIBODY

With a progressing superiority of the invading organism over the natural localizing and disposal mechanisms, the host would eventually be overwhelmed unless other defenses could be marshalled. To meet the emergency, the cellular agencies which produce globulin, presumably for the detoxification of harmful substances, come into action. Fortunately all globulin is potentially antibody. The exposure to virulent organisms and their products results in the production of antibody globulin specifically designed to combine with them, with the result that the pathogen is essentially reduced to a nonpathogenic state and is rendered susceptible to ingestion and digestion by the phagocytic cells if the latter are still functionally effective; or circulating toxin is neutralized directly by the antibody. This synthesis of specific globulin by reticulo-endothelial cells and lymphocytes, which rapidly permits the invaded body to assume the offensive, is one of the most dramatic episodes in biology. The specificity of action is also remarkable. Antibody produced against infection with Type I pneumococci acts promptly upon those organisms by combining with capsular polysaccharide but does not affect organisms of Type II. Polysaccharide which has effused into the exudate also is neutralized and its interference with phagocytosis is eliminated. As a result, the macrophages can begin their "mopping up" of the inflammatory mate-



rial, further extension is limited and recovery begins. The virulent organisms largely disappear from the body, although a few may find harborage in limited areas such as the spleen; or they may possibly survive by adaptation in a degraded avirulent stage in which they are unencapsulated and do not attract the specific immune antibody.

The antibacterial antibody developing from direct experience with infection or immunization differs from that found in the serum of inexperienced individuals—the natural antibody. The former is sharply specific for the antigens involved, it arises in recovery, it is associated with specific immunity, it is generally more abundant, it can be transferred to normal animals and furnish protection, and it is comparatively resistant to heating and less dependent upon complement for activity. It has been suggested that the differences between natural and immune antibody are only quantitative. Topley and Wilson (1946), however, clearly present the alternate interpretation that the similarities exist because of the affinity of the two types of antibody for the same chemical groupings of the organism but that the affinity of immune antibody is direct and specific, whereas that of natural antibody is incidental and more remote chemically. The concept that they differ qualitatively appears to be more in keeping with the bulk of evidence.

#### THE BEHAVIOR OF SPECIFIC ANTIBODY

For a considerable period in the development of knowledge of serologic phenomena, agglutinins, precipitins, bactericidal and phagocytosis-promoting antibodies were thought to be different components of serum. Under Ehrlich's influence the number of fractions postulated multiplied rapidly in order to meet the varied systems. As greater study of the physicochemical conditions of reactions took place and knowledge of the nature of the interacting

substances grew, the concept developed that the various reactions obtained with antibody are manifestations of the same antibody combining with a bacterial component or chemical compound under different conditions. Thus, in the case of the virulent pneumococcus, the specific globulin fraction combines with the specific capsular polysaccharide, and agglutination of the bacterial particles takes place; the sensitized organisms can also be phagocytized and digested by polymorphonuclear cells so that an infected animal which is furnished antibody will recover or a normal animal given antibody and organisms is passively protected. On the other hand, if the capsular polysaccharide is in solution, and if it is mixed with the same immune serum, there is a precipitation of antibody-polysaccharide complex which follows the same general rules as agglutination. Or complement is used up during the process of antibody combining with antigen. The skin of the immune animal will show a reaction if the specific carbohydrate is injected intradermally. A guinea pig previously given the anticarbohydrate antibody will exhibit anaphylaxis when the carbohydrate is injected intravenously. Or the micro-organism itself grown in the presence of this antibody will lose its capsule. And the mere injection of purified polysaccharide into the human gives rise to antibody which possesses all these capacities. The unitarian thesis which maintains the identity of the antibody engaging a specific antigen in the different reactions is well illustrated by these examples. The very fact that the same globulin and polysaccharide do participate in such varied performances is an excellent illustration that the antibody derived as an emergency aid to the infected body can behave in a variety of systems with one objective—to combine with, and to function in the removal of, the bacterial component essential to continued pathogenicity and virulence.

The situation with pneumococci, however,



is in some respects an optimistic oversimplification, for up to the present comparatively few of the bacterial systems have yielded equally clear parallels. Certain Gram-negative organisms, such as the cholera vibrios, undergo dissolution or lysis in the presence of immune antibody and complement. Antibodies to erythrocytes will agglutinate them, and, if complement is added, the cells are lysed. Antitoxin will combine and flocculate with toxin in vitro or neutralize it so as to give protection to an animal receiving the mixture. It is quite obvious that chemical and anatomic structure of the foreign cell involved is an important factor in determining the result of antibody action.

#### MULTIPLE ANTIBODIES TO A BACTERIUM

On the other hand, since bacteria are complex organisms, the antibody response is not ordinarily limited to one component, even though a single superficial structure may dominate the immune mechanism. The Group A, beta hemolytic streptococci produce a soluble erythrogenic toxin responsible for the toxemia and rash of scarlet fever; they possess group-specific C polysaccharide, type-specific proteins M and T, hemolysin; fibrinolysin, an enzyme dissolving fibrin clots; a leucocidin which damages leukocytes; hyaluronic acid which may increase tissue permeability, and basic nucleoprotein. In response to infection, antibody develops against each of these components which contribute to the organism's pathogenic activity. The character of the disease produced is undoubtedly influenced by the comparative activity of these factors, but the principle protective immunity against infection resides in antibodies to the type specific M protein which probably act on the surface of the organism. In addition, Gay (1935) and his associates have presented evidence that mononuclear cells are resistant to leucocidin while polymorphonuclear cells are not and that collection of the macrophages is an important contri-

bution to resistance against streptococcal infection. Examples of the complexity of antibody response corresponding to bacterial activities are readily multiplied.

With typhoid bacilli the flagellar H antigen, the somatic, toxic O antigen and the surface Vi antigen, all elicit antibodies in the course of typhoid fever, and antibody to the Vi antigen is apparently the one of greatest significance in protection. Nevertheless, each has a different immunologic meaning, and resistance tends to increase as the respective antibodies accumulate in the blood and bacteremia diminishes, even though the clinical disease is still under way. Relapses may occur even though these antibodies have developed, indicating that antibodies in the blood do not always suffice for the elimination of organisms from tissues. And the convalescent carrier of virulent typhoid organisms in the gall bladder is a commonplace.

#### RESISTANCE OF INTRACELLULAR ORGANISMS

Much of the present discussion has been concerned with the function of antibody in preparing organisms for phagocytosis, digestion and destruction either in the polymorphonuclear cell itself or in the mobile and fixed macrophages which may take up the organism or may dispose of the polymorphonuclear cell and its bacterial cargo. Consequently, the protective effect of the phenomenon has been stressed. Nevertheless, attention has also been directed to the fact that the presence of organisms in a cell does not imply that they are of necessity destroyed. In fact, many organisms appear to be maintained within cells of the body. Nor is it clear that in all instances this is a purposeful defensive action of the host rather than a selective adoption of environment by the invader. Rous and Jones (1916) demonstrated that phagocytized typhoid bacilli were protected from the action of cyanide and that erythrocytes ingested by polymorphonuclear cells did not undergo

lysis in the presence of a hemolytic serum. It is commonly accepted that antibody does not ordinarily penetrate the cells. As a result, organisms which can survive in cells without rapidly destroying them are not subject to the effect of antibody leading to their destruction. Moreover, the supernatant fluid of cellular exudates is said to contain a substance which inhibits intracellular digestion (Opie, 1905). It seems quite likely that in tuberculosis, typhoid fever, brucellosis, and in numerous other instances, the persistence of infection is associated with continued intracellular growth or survival. Under these circumstances the possibilities also exist that the proper antigen leading to staunch immunity may be destroyed within the cell, does not readily escape to stimulate immune response, or that, in contrast to those organisms against which phagocytosis is most effective, the determining antigen is less readily available at the surface for antibody union. It is of interest to note in how many instances the organisms which persist under these circumstances are Gram-negative—a group commonly possessing a toxic component in the cell body (endotoxin). The viruses as a whole are accepted as intracellular parasites which require the medium of the living cell for propagation. After gaining entrance to their specific cellular harborage they are little influenced by antibody except as they cause cellular destruction, as with yellow fever and influenza, or require transport to new cells. Certain of them which persist in proliferating tissue are slowly affected.

#### CELLULAR FUNCTIONS IN RECOVERY

Attention has been repeatedly called to the fact that the emergency development of specific antibody tends to enable the now immune animal to behave toward pathogenic organisms as the normal animal does toward harmless invaders. Nevertheless, the appearance of antibody does not correspond exactly with termination of clinical disease.

The injured tissue must be removed by the macrophages; exudate and organisms undergo digestion and removal and healing takes place. These are functions which, too, require active participation of cellular components.

That the cell of the immune animal is significantly altered from its natural state has not been well established. Nevertheless, the idea has persisted that experience results in a tuning up of the cells so that they respond more effectively to the same stimulus. Gay and Morrison (1923), Cannon and Pacheco (1930), and others have described an early large outpouring of mononuclear cells in the areas of the experienced host subjected to reinvasion. This suggests that the entire process is foreshortened so that the polymorphonuclear stage is brief and the mononuclear cells are mobilized more readily. Lurie (1939) has reported limited studies interpreted to mean that the mononuclear cells of the animal with acquired resistance to tuberculous infection have increased capacity to dispose of the homologous organism and also of carbon or colloid particles and staphylococci. It is not unreasonable that cells mobilized under protective circumstances should have a heightened capacity to dispose of foreign material. Moreover, the greater sensitivity of cellular response is seen in the fact that antibody titers tend to decline with the passage of time after active infection, but a second stimulus of the same antigen then induces an outpouring of antibody from the cells in an accelerated manner, frequently to a higher level than originally attained. Even infection by another agent may also result in a limited increase in antibody to the first. The globulin-producing cells are potentiated to ready production of antibody. In some instances the suggestion has been made that the cells in the area of infection actually produce antibody and this may be the case if lymphoid tissues or histiocytes are available in that area. However, evidence that local production of anti-



body is a major influence is far from complete.

An accelerated inflammatory response to antigens previously experienced is a common sequel to infection with many organisms. Since this hypersensitivity or allergy has been observed to occur in the animal which has developed resistance, the tendency has been to consider one the necessary accompaniment of the other. It has especially been true in tuberculosis where the development of the tuberculin reaction is rather constantly a response to infection. This subject is exhaustively considered by Rich (1944). The prime effect, however, of bacterial hypersensitivity through its speeding up of response is so to intensify the reaction of the body that small numbers of organisms cause an exaggerated response. Whether this is beneficial to the host will depend upon the particular agent involved.

Study of the phenomenon has been largely limited to reactions measured by crude bacterial proteins, fractions whose significance as essential antigens in inducing immunity is quite obscure. In consequence bacterial hypersensitivity is commonly considered with respect only to proteins. The modified state is intimately related to some modification in cells as shown by the fact that the reactivity is not transferable to a normal individual by serum of the hypersensitive, but Chase (1945) has accomplished the transfer with cells of sensitized animals. Moreover, the cells of a hypersensitive animal are directly injured by exposure to the protein material.

The differences in time of appearance and character of cutaneous reactions to different fractions of pneumococcus illustrate significant points. Reactions to the "nucleoprotein" can be elicited by injection into the skin of most adults. It is of the flat, red "tuberculin-like" nature developing gradually to a peak in 24 hours after which it slowly disappears (Tillett and Francis, 1929). In the course of an acute infection such as pneumonia, reaction to nucleoprotein fails to occur, but the capacity returns in convalescence. Precipitins to the bacterial material are, however, demonstrable in the serum throughout the entire course of the infection. Reactions to tuberculin may be similarly diminished or sup-

pressed at this time. It is also known that reactions to streptococcal protein can be neutralized by serum taken from individuals at the time of the depressed activity. These facts demonstrate that acute illness modifies the capacity of tissues to respond to bacterial protein in the same way as in health. But there is no obvious relation between that reactivity and specific immunity. Reactivity to bacterial protein of this sort is, nevertheless, a product of accumulated experience.

The somatic polysaccharide, or "C" substance, in contrast, does not cause reactions in the skin of normal individuals, but within 24 hours after the onset of illness cutaneous reactions can be induced. The reaction is biphasic, exhibiting an immediate wheal and erythema followed by an edematous but flattened reaction reaching its height in six to 12 hours. This reactivity persists until convalescence, when it disappears. The serum of the actually ill patient contains an abnormal albumin, not a typical antibody, which precipitates in the presence of "C" substance and which disappears with recovery. It is of interest, however, that in severely ill and fatal cases the cutaneous reactivity may disappear, although the precipitating activity of the serum remains, indicating a failure of tissue participation (Abernethy and Francis, 1937). The same tendency to cellular failure has been noted when phagocytic activity of whole blood has been examined through the course of severe natural or experimental pneumonia. In this instance again the significance of the reaction to immunity is not apparent; nor is it specific in that reaction to pneumococcal "C" substance occurs in other infections.

The capsular polysaccharide does not elicit a cutaneous reaction in the acute stage of illness. With the beginning of recovery and the appearance of type-specific antibodies, however, an immediate wheal and erythema reaction, so-called anaphylactic type, can be induced by specific polysaccharide of the type corresponding to that of the infecting pneumococcus (Tillett and Francis, 1929). This form of reaction is an indication of firm, specific immunity, but that cellular participation is required is seen by the fact that cases progressing to a fatal outcome may lose their capacity to react even though specific antibodies are present (Francis, 1933). That an anaphylactic reaction may be evidence of immunity is probably incidental to

the fact that the important antibody is anti-carbohydrate.

It may be worth while to call attention once more to the probability of failure in proper physiologic function of cells as constituting an important factor in the problem of chronic intracellular infections and in the relapses which commonly occur in them. In brucellosis, where measuring of phagocytosis is used to gauge the progress of the disease, it has been said that a sharp decline in this capacity frequently heralds a recurrence of acute illness. In chronic virus infections such as psittacosis and lymphocytic choriomeningitis the same influence may be at play in their native hosts. In this connection it must be recalled that while we speak of antibodies of the serum, they are not limited to the blood. Rather they are with other proteins in the intercellular fluids and exude into secretions and exudations. That most of these reactions require participation of both cellular and humoral influences is clear. It seems to a large extent that the serologic factors mediate the activity of the cells. There are instances, however, in which combinations of cells from the immune individual with his serum are said to be more effective than mixtures of that serum with normal cells. Jawetz and Meyer (1944) point this out in immunity to plague, again suggesting that the reactivity of the cells is modified to some extent.

The idea that specific immunity can reside in cells alone has had a long career. It persists most stubbornly in those diseases such as syphilis, tuberculosis and leprosy, in which the immunologic mechanisms have not been clearly demonstrated, but it has steadily withdrawn in the face of increased knowledge. That cells can be refractory to injury is true, but, in general, there is little specificity involved. At present there is no acceptable example of specific cellular immunity unrelated to serum antibody.

## IMMUNITY

### THE IMMUNE STATE

The foregoing discussion has sought to follow the broad lines of the host's behavior from the beginning of his experience with a bacterial agent to recovery from active infection by a pathogen. Specific antibodies have developed, the injury is being repaired and the individual exhibits a state in which he is able to encounter the same organism and dispose of it without undergoing the injury and reaction of active disease. This state of physiologic education is called immunity, offering the implication of complete protection or freedom from further ill effect. Experience has clearly demonstrated that in many instances this is not the case, or is for a limited period only. Consequently, terms such as degrees of immunity and resistance are employed to indicate the relativity of immunity.

### VARIATIONS IN IMMUNITY

Various schema have been devised to illustrate the theoretical results of exposure at different levels of resistance to different degrees of virulence of an organism under conditions of presumably constant dosage. Complete immunity resists organisms of all degrees of virulence; at lesser levels of resistance, the virulent organism may be capable of establishing infection ranging in severity from inapparent infection to a second clinical disease.

In these intermediate grades of resistance the organism may become temporarily established, but the entire reaction of the host is more efficiently mobilized. The procession of events may be fundamentally similar to the first response, but the organisms are held in place, microphages are quickly followed by macrophages and repair takes place in rapid succession. The fixation of the organisms so as to limit their



invasiveness and enhance their disposal is gained by the ability of specific antibody to unite with the organism and to enable the other physiologic agencies to carry out their functions.

Even a minor resistance has a beneficial influence. The different levels of resistance observed in individuals may be related to the severity of the first exposure and reaction; or to the fact that resistance and antibody levels tend to decline as the interval after infection increases; or to the fact that inapparent infection may furnish the antigenic stimulus for increasing a resistance earlier acquired. The importance of the last influence has become increasingly recognized as serologic procedures for many diseases have improved and since the frequency of exposure to infection has been reduced by the application of control measures to the disease. As the amount of infection decreases the opportunity for mild immunizing experiences decreases and the population as a whole is more susceptible than earlier generations. Immunologically, the fact that infection occurs is the important item rather than that the infection should take the course, so often a physiologic accident, of what is termed the "typical clinical disease." Nevertheless, since disease is the problem with which much of medicine is concerned, it is the ability to avoid clinical illness which measures the efficacy of an immunity. It is for this reason, too, that knowledge of the actual conditions at play in the different disease processes is important in interpreting the efficacy of immune responses.

Certain bacterial and virus diseases with short, sharp courses of infection are followed by a solid, relatively permanent immunity. Perhaps the most illustrative are diphtheria and scarlet fever on the one hand and smallpox, measles or yellow fever on the other. In each of the first instances the agency concerned is a bacterial exotoxin which is transferred through the blood and

attacks specific tissues directly. The others are virus infections in which blood invasion is essential, and these agents also specifically attack cells.

In contrast, despite the fact that specific antibodies develop, recovery from pneumococcus pneumonia does not insure a permanent resistance. In fact, Finland and Winkler (1934) demonstrated that the frequency of Type I pneumococcus pneumonia in individuals who had previously recovered from that very disease was no different from the incidence of Type I pneumonia in the general population. Gonorrhea and dysentery may be incurred repeatedly. So may influenza and herpes. And even though antibodies to the erythrogenic toxin of streptococci may persist so as to prevent scarlet fever, the organism can still cause pyogenic infections. Tuberculosis or brucellosis may continue into a chronic disease as a state of comparative resistance develops in the host. The same is true of certain virus infections.

The duration of immunity to certain species of organism is also limited by the fact that different immunologic strains or races may be uninfluenced by specific immunity to a single one of them.

These illustrations serve to demonstrate the great variations in the character of resistance which accompanies recovery from acute infectious disease. And since the capacity to produce antibodies persists for an indefinite period, the differences are not related to loss of that function. Lymphocytes and reticulo-endothelial cells continue the production of globulin with linkages effective against the bacterial antigens. If the infectious agent is destroyed and infection is eliminated, the formation of specific globulin may gradually decline through lack of demand, much as in the case of adaptive enzyme production. If, however, the organisms remain active and the stimulus reaches productive cells, specific antibody continues to be synthesized.

PERSISTENCE OF IMMUNITY, ANTIBODY  
AND INFECTION

But the production of immune antibody is not dependent upon the constant presence of an infectious antigenic stimulus in the body, as is seen by the fact that a single inoculation of minute amounts of a purified protein, such as egg albumin or a carbohydrate, may be sufficient to set off the production of specific antibody for the lifetime of an individual. On the other hand, one can point to the complement-fixing or flocculating properties of syphilitic serum with nonspecific lipids as continued evidence of infection so that treatment is sometimes directed more by the serologic test than by clinical observation of the disease. The prolonged immunity observed following certain virus diseases has been ascribed by some to a continued residence of virus in the cells of the recovered animal where it continues its stimulation, but evidence to support the thesis is unconvincing.

The behavior of the host with continued infection has, however, resulted in the term, infection-immunity. Reinoculation of the same agent into the infected animal ordinarily does not give rise to the same sequence of clinical events and inflammatory signs as follow the initial introduction. Since the defenses of the host are already preoccupied with its active infection it appears indifferent to a reasonable new inoculum. It is scarcely immunity but rather a lack of normal reactivity to the same organism and is better called premunition. The fact that chronic infection is commonly accompanied by continued high levels of antibody and by premunition has led to the illogical inference that prolonged immunity and antibody production require persistence of the causative organism.

It has become apparent that antibodies must be directed against the proper antigens of the bacterial cell to furnish effective resistance and that antibodies to other com-

ponents may be of little significance. Even when the correct ones are present, it is clear that the presence of antibodies is not constantly synonymous with immunity—a fact frequently overlooked by the serologist.

RELATION OF IMMUNITY TO THE PATTERN  
OF INFECTION

An understanding of the variations in resistance observed after different diseases, assuming the presence of desired specific antibodies, can be best obtained by an interpretation of the physiologic pattern involved in the infectious processes, the pathogenesis (Francis, 1947). Antibodies in the general circulation should be most effective in those diseases in which direct cell injury is caused by agencies requiring transport in the blood stream, as in diphtheria or measles. Infections of superficial tissues like the intestinal mucosa in dysentery, the mucous membranes in herpes or the respiratory epithelium in influenza are probably less influenced by circulating antibody *per se* than by antibody available in the secretions about the respective tissues. The superficial extracellular localization of pneumococci or streptococci would tend to evade the influence of circulating antibody except as it becomes available in inflammatory fluid or limits progression of the organisms in the blood. This concept, that to be most effective antibody must be available at the initial site of infection where the organism can be met and sensitized by immune globulin for disposal, has grown in recent years since more attention has been given to physiologic processes in infection. The decisive factor is the amount of antibody available at the portal of entry or at some point where it encounters the organism in the developmental stages of infection and thus gives immunity to the body as a whole. The significance of the level of antibody in the blood would lie in the amount which



could be furnished to the local zone of invasion.

Intracellular infections, it has been pointed out, may evade the action of antibody except as cells are destroyed and the agents dislodged from their protected environment. The conflict becomes one of attrition with varying balances in equilibrium between a rapidly developing parasitism and the effort of the host toward extinction of the agent.

#### CELLULAR FUNCTIONS IN IMMUNITY

The continued need for normal cellular participation in the maintenance of immunity has been indicated, and reference has been made earlier to examples in which cellular participation fails. It appears probable that the increased tendency to acute infections in the aged or in the debilitated may be associated with a similar disturbance. Impaired physiologic function of cells may result in diminished formation of immune globulin and in reduced phagocytic clearance. In those instances relatively non-pathogenic organisms or those which have become well-adjusted members of the normal flora tend to penetrate the weakened defenses. And invasion by one's own parasites is likely to produce a prolonged serious illness since these organisms have had previous experience with the host's defenses which now are ruptured. The severity in older individuals of pneumonia caused by Type III pneumococcus, an organism frequently carried in the respiratory tract, is illustrative. Infections with *E. coli* can be considered in a similar light.

Kahn (1936) has spoken of the capacity of cells to combine with or to fix an antigen or organism as an essential, specific mechanism of the immune animal comparable to antigen-antibody union. There is obvious reason to accept the principle that a variety of cells play a role in the fixation of injurious materials, but it is difficult to see

clearly the evidence that all tissues possess that as a specific activity. At the same time, the argument that tissue immunity exists because resistance develops before antibodies are demonstrable in the blood is not valid, because antibodies of the circulating blood are presumably the excess of what is needed in the areas of immediate involvement. Tissue immunity, independent of antibody participation, is still to be adequately demonstrated. Nevertheless, it is conceivable that the intracellular functions can be heightened so as to aid in the extermination of the parasite ensconced in the cell.

Discussion of cellular resistance to infectious agents in recent years has been attracted to the phenomenon of interference observed especially among viruses. The introduction of one agent within an interval of several hours of introduction of another may result in the blocking of the capacity of the second one to establish itself. In the monkey, Hoskins demonstrated that the mouse-adapted strain of yellow fever virus which no longer produced hepatic injury could protect against the fully virulent strain of the same virus inoculated at the same time. The number of examples has expanded tremendously to show that there need not be an antigenic relationship between the interfering agents, and it is not sharply specific. But it is not associated with antibodies developing from the infecting agents. It appears that the blocking agent may attach to the component of the cell which is essential to the growth of the other and thus preclude the latter's establishment, or as shown with bacteriophagy, the permeability of the cell may be altered to prevent entry of the second agent. It is not specific immunity, and it seems less desirable to consider it as cellular immunity than as another example of less specific refractoriness—perhaps not too different from the interference with infection by viruses created by physical or chemical irritations.

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## 6

# The Allergic State

### INTRODUCTION

Allergy deals with a wide variety of pathologic phenomena, some of these being so different from one another that there is no immediate suggestion of a common basis, e.g., serum sickness, food allergy, poison ivy dermatitis, anaphylaxis, tuberculin sensitivity. During the course of this discussion, an effort will be made to bring out the fundamental relationships and to interpret them in the light of immunologic processes. The task is large and will hardly allow consideration of the relation of the allergic state to the pathogenesis of disease or to immunity. Various aspects of these questions, which are highly controversial, are discussed by Tytler (1930), Opie (1936) and Rich (1944) and are referred to in Chapters 4 and 12.

A rather simple terminology will suffice, and in line with the currently broader interpretation of older terms\* we shall use the term "allergy" in nearly its original sense, i.e., an altered capacity to react, as judged from previous experiences of the same individual or from the experiences of other individuals of the species; furthermore, it is an alteration that is specific, usually reflecting prior contact (although not always evident) with the same material or one closely related to it chemically. This

definition must exclude the occasional cases of unduly high response to physiologically active materials when the response differs solely in intensity from the pharmacologic effects produced in other individuals.

In consequence of the many attempts at classification made prior to an understanding of basic mechanisms, the prematurely adopted concepts have required subsequent modification. At the same time, the former terms have been retained and defined anew, though at best they are vague in inherent connotation: allergy ("altered reactivity"), idiosyncrasy (eccentricity, "peculiar mixing together"), atopy ("not in place," "a strange disease"), anaphylaxis (devoid of protection) and hypersensitiveness. The word "allergy" was suggested by von Pirquet in 1906, in generalizing the implication of his studies with Schick on human serum sickness, as a term to cover all changes induced in the state of reactivity in consequence of contact with any living thing or inanimate substance. By gradual and unguarded changes (of which one stemmed from a later limitation by von Pirquet), we arrive at the situation today where some few "allergists" may consider that "allergy" is a term that rejects, rather than includes, both the hypersensitiveness of anaphylaxis and the tuberculin type of sensitivity. To take another example of unsettled usage, the term *atopy* was introduced to describe allergic diseases that exhibit a genetically determined susceptibility for sensitization, and, with this, the appearance of special antibodies called *reagins*; but these conditions are found to be not always inseparable and so the term *atopy* is in process of transference and is coming to connote simply all allergies of the wheal-and-erythema type exhibiting "reagins."

\*The reader is referred to the following texts: Cooke et al., 1947; Zinsser, Enders and Fothergill, 1939; Sulzberger, 1940.

On the one hand, there is a desire to start afresh and to employ different terms. The most useful, probably, are those which Roessle suggested on the basis of histologic studies, namely *normergy* as a designation for the norm of the inflammatory responses of comparable normal tissues to a given stimulus, *hyperergy* for supranormal reactivity, *anergy* for lack of normergic reactivity (owing either to the immune state or to abnormal cellular physiology); *hypoergy* has been employed as well, commonly in the sense of a lessened reactivity subsequent to a sensitivity. Still other designations are proposed from time to time for conceptual reasons or for special descriptive purposes, e.g., *pathergy*, *parallergy* and *iathergic reactivity*, and an expanded classification of allergic manifestations in such terms has been advocated by Urbach (Urbach and Gottlieb, 1946). On the other hand, there is, as mentioned, a rather ready acceptance of old terms with broadened meanings, and they are sufficient for this presentation. It will be evident that nearly all of the manifestations of allergy are instances of increased levels of reactivity (hyperergy).

The manifestations of allergy are multiple indeed, and are so various that the reactions clearly belong in different classes. We may, however, question seriously whether the basic mechanisms are correspondingly many, whether it would be reasonable to assume that the body possesses a multiplicity of entirely independent mechanisms for the recognition of substances that it has previously met with. The production of antisubstances (antibodies) in response to antigenic stimuli has been known for long, and in the specificity of antigen-antibody reactions (Landsteiner, 1945; Boyd, 1947) we see one basic principle that finds repeated expression: the patient recovering from typhoid fever exhibits agglutinins directed against the invading bacterium; again, upon recovery from clinical diphtheria the skin may be no longer reactive to certain doses of diphtheria toxin but is "Schick negative" owing to the development of *antitoxin*, which end can be attained also by injection of toxin-antitoxin mixtures or of toxoid; the hay-fever patient

exhibits special antibodies called *reagins*, which are essentially specific and are useful for diagnosis of the causative allergen; and the individual recovering from serum sickness can show specific antibodies capable of sensitizing normal skin ("reagins"), and precipitins as well.

Are there other basic mechanisms also? The answer is: we do not know. Certainly there are operative mechanisms that are not readily interpretable in terms of experience with known antigen-antibody systems. We do not understand the events in delayed-type reactions that become evident over the course of several days, such as the tuberculin reaction or poison ivy dermatitis, despite the manifest specificity; there is no correlation with circulating antibodies. In several cases it would appear that we have to deal with cell-bound, possibly intracellular, antibodies—for instance, in the sharply localized "fixed eruptions" seen at times in such conditions as formaldehyde sensitiveness of the skin, where the response to each fresh exposure occurs only or chiefly in the same cutaneous areas.

In human allergies that are known to be related to circulating antibodies, the idea is frequently voiced that antibodies can be distributed unequally among the various tissues, far more so than we are wont to recognize in laboratory animals that are sensitized via the hypodermic needle. Such a concept is embodied in the oft-heard term "shock organ," which is used to distinguish the sites of predilection for allergic reactions seen in various individuals—the bronchial mucous membrane (asthma), the mucous membranes of the eyes and the upper respiratory tract (hay fever, allergic coryza or rhinitis), epidermal cells (eczematous reactions, "contact dermatitis"), or gastrointestinal allergy, or endothelium of the superficial vessels in the upper cutis (urticaria, wheal-reacting allergies). Basically these matters are not understood, and many problems remain for solution. To some extent, the concept of a particular "shock



organ" will be an artificiality, for the observed reaction may reflect the usual route of contact with the allergen, rather than solely the existence of loci with exaggerated sensitivity. For example, a person who suffers only from hay fever under natural conditions of exposure will occasionally exhibit, instead, an attack of asthma or a generalized urticaria when his allergen is injected for prophylactic purposes.

#### TYPES OF ALLERGIC RESPONSE

Allergic reactions fall into two chief categories which are not always sharply separable—the "immediate" and the "delayed" types. These have also been epitomized respectively as the "urticarial" and the "tuberculin" types of reactions.

The reactions of "immediate" type become well developed soon after application or after adequate absorption of the corresponding allergenic substance (asthma, hay fever, "hives," certain unusual cases of drug sensitivity, gastro-intestinal disturbances). In many instances of sensitivity of this sort, circulating antibodies are demonstrable, and there is some accompanying skin reactivity which assists in recognition of the allergen by means of a "wheal-and-erythema" response in the skin where the allergen is applied. In certain manifestations of gastro-intestinal allergy, however, in which the reaction-type can be of the "early" sort, there may be no parallel skin reactivity or demonstrable circulating antibody.

The "delayed-type" reactions, on the other hand, require at least several hours after introduction of the allergen before an effect is manifest (reactions to poison oak or poison ivy, most drug sensitivities, reactions to products of micro-organisms—tuberculin, mallein, histoplasmin), and show progressive changes for two to three days or longer. In these cases, circulating antibodies are but rarely found, and such antibodies give transferred reactions only of the early or immediate type.

In our consideration of allergic manifestations, principal emphasis will be given to evidences of the participation of antibodies in allergic phenomena; and to this end instances of duplication of allergic responses in a normal individual by means of serum taken from a sensitive individual—so-called *passive transfer*—will be stressed. Illustrative examples will be taken as freely from experimental studies on animals as from man, and comparisons and contrasts between various species will be drawn repeatedly.

#### ALLERGENS

The materials giving rise to the allergic states, and thereafter producing allergic reactions, are manifold: foreign proteins, including foodstuffs, and digestion products of these; nitrogenous, probably polypeptide-like, water-soluble materials contained in pollens; pollen oils; and other fat-soluble plant extracts, including urushiol from poison ivy; fat-soluble materials such as those which may be found in chocolate; chemicals and drugs of low molecular weight, as formaldehyde, 2, 4-dinitrochlorobenzene, paraphenylenediamine, mustard gas, quinine, pyramidon, sulfadiazine; metals such as nickel; and many others besides. To these we should append micro-organisms (molds, fungi, yeasts, bacteria), dead as well as living, some of their soluble constituents and possibly certain of their metabolites, and also viruses. In infection the situation becomes complex because of progressive invasion of the host and multiplication of the invading material (allergenic excitant). There are also some physical conditions (certain wave lengths of light, chilling, and the like) to which certain individuals respond with quite real allergic symptoms (*physical allergies*).

Inspection of the above incomplete list, particularly of the nonliving materials, reveals that some of the allergenic substances are frank antigens, others (pollen extracts) can be shown to exhibit some degree of anti-

genicity, and many indeed are obviously not antigens. For this reason it may be convenient to use for all of them the term *allergen* or *allergic excitant*. So far as experimental analysis has been carried out, it seems probable that many of the nonantigens do combine with proteins or other substances of the host's tissues and assume thereby the nature of an artificial antigen, although some alteration in the original structure owing to intermediate metabolism may at times be involved.

The opportunities for exposure are quite unequal with the different classes of allergens: it is evident that many individuals make adequate contact with poison ivy or poison oak, while few acquire pollen-oil or tulip-bulb dermatitis. In very many cases sensitization will be the result of repeated exposures, and the necessary number of these varies among individuals and is indicative of differences in the host factors determining susceptibility to sensitization. Indeed, with certain allergies, such as hay fever, the genotype of the individual has been said to exert absolute control over the capacity for sensitization; this matter has not yet been subjected to adequate experimentation.

## SYSTEMIC ANAPHYLAXIS

The term "anaphylaxis" was coined by Richet (Portier and Richet, 1902) to describe the state of excessive susceptibility, the very antithesis of prophylaxis, which he was encountering in dogs undergoing intravenous injections with toxic materials, eel serum or extracts of the tentacles of sea anemones. Richet found that rather than becoming "immune" the dogs would become violently ill upon re-injection about three weeks later and often died. Independently, at about the same time in this country, Theobald Smith reported to Ehrlich a characteristic and dramatic sequence of events observed upon re-using guinea pigs that had been employed for the injection of mixtures of diphtheria toxin and antitoxin: such animals died acutely or showed severe illness if they were injected with normal horse serum.

The early observations, well supplemented by the careful studies of Ehrlich's pupil, Otto, and of Rosenau and Anderson (1906, 1908, 1909), and others after them have established that the phenomenon is the result of antigen-antibody reactions occurring in vivo. Richet's extracts were rich in antigenic foreign protein, and in Theobald Smith's case the antigenic proteins of horse serum constituted the foreign material common to both injections. The first injections had stimulated the production of antibody; the later injection had supplied the antigen needed for ready combination with this antibody.

Anaphylaxis proper, then, may be defined primarily as an acute *systemic* reaction, species-characteristic, that is exhibited by animals in the hypersensitive state upon re-injection with the same material; these systemic reactions come on soon after the injection and are definitely of the "early" type.

Regardless of the antigen-antibody system involved, the syndrome of anaphylaxis as observed in a given species of animal is, as mentioned, always the same; it differs characteristically, however, from one species to another. We shall consider first the anaphylactic sensitization and response of the guinea pig to native foreign protein, and illustrate the various aspects of anaphylaxis by studies made in this species. Then we shall examine briefly the special anaphylactic syndromes exhibited by other animals, and finally consider the theories of the mechanism. While anaphylaxis is an artifice of the laboratory, it illustrates well most of the general principles recognized in antigen-antibody-tissue relations, as will become more apparent in the later consideration of the immediate type of localized allergic reactions.

Comprehensive reviews of the subject are presented in Doerr (1929); Seegal (1935); Zinsser, Enders and Fothergill (1939); Berger and Hansen (1940); Ratner (1943); Topley and Wilson (1946); and Dragstedt (1941, 1945).



## ANAPHYLAXIS IN THE GUINEA PIG

In the guinea pig, the anaphylactic state is readily established through a single injection of soluble foreign protein, usually given subcutaneously. Small amounts are adequate—0.1 cc. down to 0.0001 cc. of horse serum (0.000001 cc. at times, Rosenau and Anderson), or 0.1  $\mu$  to 1 mg. of crystalline ovalbumin. With some materials that are less antigenic than native proteins, several preparatory injections may be necessary. The symptom-complex known as anaphylaxis will then be demonstrable as typical, acute shock when a second injection of the antigen is given by the intravenous route 10 to 21 days later. The releasing injection should provide an amount of antigen sufficient to establish a not inappreciable concentration in the circulation, as 0.05 cc. to 0.5 cc. of horse serum, usually put into the blood stream in the course of 30 to 60 seconds. As a less satisfactory alternate to the latter, larger amounts may be given intraperitoneally, in which case shock, often not so acutely manifested, starts when sufficient of the antigen has been absorbed.

Within the following minute, restlessness is evident; then the hair, especially at the nape, bristles; feces and urine are often voided; the animal scratches at the mouth with a wiping motion of the forepaws; it coughs, arches its back and raises its head with obvious dyspnea; it gives a series of jerks, sways, goes into violent tonic and clonic convulsions and falls over, with death ensuing after a few shallow gasps; there is terminal cyanosis. All this occurs often within a period of three to five minutes: postmortem search reveals firm inflated lungs, an actively beating heart, evidences of visceral congestion, active peristalsis and decreased coagulability of the blood.

Obviously, lesser degrees of anaphylactic sensitivity than the rapidly fatal form described here are to be encountered. Minimal symptoms include scratching, defecation, and one or more of the characteristic coughs; dyspnea may be transient or not evident; there may be a brief elevation of body temperature rather than the decrease usually noted.

There is another form of shock ("protracted shock") in which dyspnea and bronchoconstriction are much less prominent or even absent: this can result from subcutaneous or intraperitoneal injection of antigen into the sensitized animal or, rarely, from intravenous injections (Williamson, 1936). Instead of shock that is explosive in character, there is a profound depression, at times comalike and often with copious tearing, accompanied by marked drop in body temperature; this can last from between 15 minutes up to several hours before death or recovery. When death occurs, one observes instead of a ballooning of the lungs, edema and hemorrhages of the lungs and congestion of the viscera, especially the liver. Other data, also, suggest a participation of the liver (Winter, 1945).

Where the shock persists for some time, many features that occur in acute shock as well are to be seen more plainly. These include changes in blood pressure, which after an initial rise falls steadily during the entire period of shock, a fall in body temperature (sometimes as much as 8° or 9° C.), the almost complete loss of coagulability of the blood, which has been attributed to release of heparin from the liver, a leukopenia, a marked decrease in numbers of blood platelets, and often a diminution in the complement titer of the blood.

The symptomatology of acute shock in the guinea pig is referable to a general contraction of smooth muscle; this explains the cutaneous pruritis and bristling of the hair, the defecation and emicturition and the acute death as well, for the contraction of the smooth muscle around the secondary and tertiary bronchioles—so prominent an anatomic feature in the guinea pig—occludes the air passages through infolding of the bronchial mucosa and results in death by suffocation, with air locked in the lungs. The stimulus for the contraction of smooth muscle upon antigen-antibody interaction may well be a sudden release of histamine and other physiologically active substances (cf. Campbell and Nicoll, 1940).

In employing anaphylaxis for determining the immunologic relationship of proteins, it may be pointed out that, as in other serologic tests, cross reactions between

related antigens and antibodies are exhibited, and it is of course necessary to take account of quantitative relationships in order to show that the effect of the homologous antigen is actually maximal, a matter not seldom overlooked despite the danger of misinterpretation.

**Organ Anaphylaxis: Schultz-Dale Test.** The features of anaphylaxis encountered in the intact, sensitive guinea pig are met with also in the individual organs tested in situ or after isolation and perfusion. Upon contact with the specific antigen, uterine muscle tissue or intestinal segments contract; in the lung, either free or in the isolated heart-lung preparation of Starling, bronchospasm and occluding constriction may be demonstrated; blood vessels exhibit constriction; strips of gallbladder contract; the isolated heart exhibits an increased rate of beat, arrhythmia and constriction of the coronary arteries (Wilcox and Andrus, 1938; Andrus and Wilcox, 1939); and the like. All these responses are transient, the tissues soon returning to the normal state while antigen is still present. In the same fashion, guinea pigs that are only slightly sensitized soon start to recover after a shocking dose has been injected.

There are several ways in which "organ anaphylaxis" can be especially useful, and accordingly the anaphylactic test is often carried out not by observation of the systemic response of the entire animal but simply by testing uterine strips or intestinal segments taken from the animal (Schultz, 1910; Dale, 1913). By this means it becomes possible to demonstrate specific desensitization, that is, the complete loss of anaphylactic (but not physiologic) reactivity of the tissue following one maximal response to specific antigen, and through this demonstration to rule out non-specific causes for shock. Then, too, one may test several strips of tissue from a given animal with different antigens. And in instances where sensitization is minimal and systemic shock would be low grade,

definite anaphylactic effects can be observed.

In the Dale test, often called the Schultz-Dale test, the uterine horns of young, non-pregnant female guinea pigs (250 Gm. weight) are used and accordingly females are often chosen for active sensitization (or for *passive anaphylactic sensitization* by injection of antibody-rich serum, as described later). The uterine horns are suspended individually in warm, oxygenated baths of physiologic solution suitably balanced as to calcium concentration, with the basal portions fixed and the fimbriated ends connected with writing levers. When the normal muscular rhythm is established, antigen is added to the bath. After a latent period of approximately 30 seconds, the tonic contraction of the muscle raises the lever; the excess antigen is then removed by draining the bath, and new bath fluid is provided; once again under approximately normal muscular rhythm, the horn makes contact with the same antigen, this time without effect (desensitization); finally, showing that the lack of response is not due to progressive disfunction of the muscle in artificial environment, a test is made with a nearly threshold concentration of histamine [or pilocarpine or acetylcholine or mecholyl (acetyl-beta-methylcholine chloride)], by which a typical contraction must be demonstrable. (The desensitization, of course, pertains only to the antibody mechanism for inducing further muscle contraction.) This series of events is illustrated in Chart 2, where a submaximal sensitization



CHART 2. Graph of Schultz-Dale test. Contraction of a sensitive uterine horn in the presence of specific antigen is shown.

The guinea pig was sensitized passively by the injection, 24 hours before, of rabbit antiserum prepared against ovalbumin. Key: A, addition of antigen (ovalbumin) to the bath; X, complete replacement of bath fluid; Hist., histamine added to secure a concentration of 1:20,000,000.



has been established passively by means of immune rabbit serum. The uterine horns after anaphylactic contraction due to antigen-antibody interaction have relaxed gradually even while antigen is still present. According to Nicoll and Campbell (1940), strips of intestine, first used by Schultz in 1910, can be handled in the same way with greater facility and equal sensitivity.

The relation of the visceral peritoneum to the antigen-antibody reaction which stimulates the underlying smooth muscle of the uterine horns or intestinal segments is somewhat obscure. The latent period before the muscle contracts is usually much the same for all antigens. Apparently the visceral peritoneum may function as a barrier to an antigen of very high molecular weight, tobacco-mosaic virus, but not to *Limulus* hemocyanin having a molecular weight of about 3,000,000 (Seastone, Loring and Chester, 1937; Kallos, 1938).

**Passive Transfer.** The antibody which leads to the anaphylactic state is present for a time in the serum in sufficient amount to allow passive anaphylactic sensitization of normal guinea pigs. Following the transfer of serum in amounts of the order of 2.0 to 4.0 cc., taken 8 to 14 days after the preparatory injection, and an incubation period of perhaps 18 hours, the recipient animal acquires temporarily the specific sensitivity of the serum donor, and will exhibit anaphylaxis in the manner of actively sensitized animals when an intravenous injection of the antigen is made within the next few days.

Anaphylactic sensitization results from the injection of any antiserum prepared in the guinea pig. The amount of guinea-pig antibody that is required to lead to fatal anaphylactic shock has been measured quantitatively by Kabat and Boldt (1944) for an ovalbumin-antiovalbumin system and is extremely small—0.005 to 0.03 mg. antibody Nitrogen or less than 0.2 mg. antibody-globulin. With especially prepared guinea pig antisera, amounts as little as 0.2 cc. may contain this amount of antibody; usually much greater volumes of antiserum—2 cc. or more—are needed.

Guinea-pig tissues can be sensitized likewise by antibody produced in the rabbit (Chart 2), a species that yields antisera of

high titer. Therefore, immune rabbit sera are used most often for passive anaphylactic sensitization, since the antibody content of guinea pig antisera is usually low. The minimal amount of rabbit antibody necessary to induce fatal anaphylaxis has been measured quantitatively by Kabat and Landow (1942) and is of the same order of magnitude (about 0.03 mg. antibody Nitrogen) as is required with guinea pig antibody; however, this may be contained in as little as 0.02 cubic centimeters of rabbit immune serum. Other relationships due to use of more than the minimal amount of sensitizing antibody have been studied as well (Kabat, 1947).

Isolated normal tissue (uterine horn) may be prepared to undergo anaphylactic responses by being bathed in antiserum (Dale, 1913; Hartley, 1939; Kulka, 1943).

The guinea pig is essentially insusceptible to sensitization by antibody produced in the horse, cattle, chicken or rat; besides rabbit immune sera, mentioned above, some human sera can establish passive anaphylactic sensitization—serum-sickness sera (Longcope and Rackemann, 1918; Tuft and Ramsdell, 1929), some specimens of serum from asthmatic cases (Ramsdell, 1930), etc., while other human sera, containing only "reagins," do not do so.

It has been found advisable repeatedly to allow an incubation period of some hours (2 to 18) between the giving of antibody and the subsequent challenge of the guinea pig, and from the start this was interpreted as being the time required for antibody to become fixed to tissue cells. Indeed the process involves more than a single step (von Fenyvessy and Freund, 1914), for the amount of antibody necessary to determine the anaphylactic state can be taken up from the blood stream within an hour, long before the sensitivity itself can be demonstrated. The sessile state of the anaphylactic antibody was considered beyond question when it was found that the perfused uterus of an actively or passively sensitized guinea pig can react in the Schultz-Dale experiment (Dale, 1913). This argument was weakened considerably, however, when Freund and Whitney (1928), in studies with *rabbits*, found that antibody diffuses slowly into the uterus and skin and is then beyond the reach of perfusion fluid, presumably in the intercellular spaces; but it is readily recovered when the perfused tissue is ground with sand. These workers suggest that the incubation period seen in passive anaphylaxis represents the time necessary for the establishment of an

intimate relation between the antibodies and the tissues.

The incubation period was cited as a telling argument in the controversy over the "cellular" versus the "humoral" hypothesis of anaphylaxis (see *The Mechanism of Anaphylaxis*). While the importance of the incubation period is hardly doubted, more recent work has shown that certain normal guinea pigs—perhaps those with a special hereditary susceptibility (Zinsser and Enders, 1936)—can undergo anaphylactic shock when antigen and antibody are injected practically simultaneously (Dean, Williamson and Taylor, 1936; Kellett, 1935). For this, the volume of immune rabbit serum must be much larger than is necessary when an incubation period is observed. Also some other animals (e.g., dogs, mice) are found to require no incubation period (Sherwood et al., 1946; Burdon, 1946).

In the guinea pig, the anaphylactic state can be "inherited" from the mother, even from an animal sensitized long before, and devoid of detectable circulating antibody at commencement of pregnancy; several successive litters may be found sensitive. This appears to be an instance of passive transfer of antibodies, as the sensitivity is gradually lost during the first 6 to 10 weeks of life. In addition, it is possible to obtain actively sensitized offspring by placental transmission of antigen when the latter is given a few days before the birth (Ratner).

**The Refractory State.** There are several procedures which, although involving various mechanisms, prevent the appearance of systemic anaphylactic shock when antigen is injected. The principles should be understood, for they are encountered as well in local allergic responses of the skin.

**ANTIANAPHYLAXIS.** If one uses, instead of the minimal antigenic stimulus required for establishing the anaphylactic state, a larger amount of antigenic material (e.g., 2 cc. of horse serum), or several preparatory injections instead of one, the anaphylactic state can be demonstrated only after the lapse of five or six weeks, and the animal is said at the end of the ordinary resting period of 3 weeks to be in an "antianaphylactic" or refractory state. The chief factor has been said to be that the guinea pig acquires too much

circulating antibody to permit of anaphylactic responses, for even while it itself is "anti-anaphylactic," its serum is capable of transferring passively the anaphylactic state to normal recipients, and its organs, perfused free of serum, respond characteristically to antigen; the antibody circulating in the blood stream would then serve as a barrier between introduced antigen and the antibody in intimate contact with the tissues, as has been demonstrated by Dale and Kellaway with organ anaphylaxis in vitro. The actual situation is probably more involved (Morris, 1936a, 1936b; Bronfenbrenner, 1944).

**NONSPECIFIC INHIBITION OF SHOCK.** Aside from the specific state of "antianaphylaxis" just mentioned, a wholly temporary and non-specific refractoriness can follow the administration of drugs that depress the animal's physiologic reactions during the combination of antigen and antibody. For this reason it is usually wise to avoid anesthesia in demonstrating anaphylaxis. The effect of anesthetics is, however, of low order. Among the drugs we may mention ether, chloroform, chloral hydrate, atropine and adrenalin (epinephrin), the last-named being particularly valuable for therapeutic use in human allergic diseases: it both relaxes smooth muscle and contracts some of the peripheral vessels. In addition to these, there are now available wholly new groups of drugs, collectively termed "antihistaminics" (page 149), which in adequate dosage can block, largely or entirely, the appearance of anaphylactic symptoms. Many other substances that depress anaphylaxis, some with modes of action obviously different from that of the drugs just mentioned, are cited by Hill and Martin (1932).

Then, too, there are some special procedures which result in a quite temporary nonspecific "desensitization" of the guinea pig, such as the intravenous injection of normal homologous or heterologous serum (Friedberger; Kellaway and Cowell; Morris, 1936a). The mechanism is not understood, but an actual loss and return of the sensitivity of smooth muscle has been demonstrated (Kellaway and Cowell, 1922). In addition, a partial or complete loss of the capacity of a tissue to react under a second antigen-antibody stimulus may exist for a time after a reaction (cf. Nicoll and Campbell, 1940; Chase, 1947).

**DESENSITIZATION.** A temporary "desensitization" was noted early in those subjects that survived shock, owing to lesser grades



of sensitivity: reinjection soon afterwards produced no symptoms of shock. Besredka then showed that introduction of very small amounts of antigen beneath the skin, given as a series of injections of increasing amounts over the course of a few hours without the production of symptoms, served to desensitize the animal and to render it immune to shock for some days. Practical use is still made of this procedure, when sensitized animals must receive further injection (Kay, 1940): this has been applied successfully to man also, but it is hazardous (Friedberger and Mita, 1912; Mackenzie, 1921; Ratner, 1943). Eventually, however, with cessation of injections the anaphylactic state becomes reestablished; *there is no known technic for effecting a permanent desensitization.*

As an explanation for the mechanism involved in desensitization (cf. Longcope, 1923), it is commonly said that the available antibody is "neutralized." This does not appear to be a final answer, and indeed in one special case—anaphylactic shock imposed on the normal mouse by nearly simultaneous injection of antigen and antibody (Burdon, 1946)—it is claimed that shock results solely from the first sequence of injections and not upon their repetition despite the additional antibody furnished.

**Inhibition and Shock by Haptens.** With the advent of artificial conjugated antigens owing to the work of Landsteiner (see page 173), it became possible to examine by anaphylaxis the serologic specificity lent to proteins by the attachment of certain types of chemical radicals, which have come to be called haptens. [There may be cited as examples of artificial conjugated antigens the products resulting from the coupling of diazotized *p*-aminophenol glucosides with the proteins of horse serum (Tillet, Avery and Goebel, 1929), or of diazotized *p*-arsanilic acid coupled to protein; the respective haptens would be uncombined glucoside and *p*-arsanilic acid.] It turned out that following sensitizing injections with such a grouping combined with one protein, fatal shock could result from subsequent testing with the same grouping combined with an entirely different protein. When now the hapten itself (aminophenol glucoside) or a simple derivative of it ("*p*-arsanilic

acid tyrosine") was injected intravenously prior to the shocking conjugate, a state of specific inhibition of shock was to be found, akin to inhibition in vitro of a precipitating antigen-antibody system, that is, the small molecule could compete successfully with the large protein complex for the antibody and so delay the requisite interaction between antibody and the full antigen. In some cases the effect appears to be a simple inhibition, with the tissue retaining its full reactivity, in others there is evidence for specific desensitization as well.

On the other hand, there are special instances in which the haptenic structure is itself capable of inducing shock in sensitized animals. Thus microbial polysaccharide haptens (from *Aerobacter aerogenes*, Friedländer's bacillus, and a yeast) were shown by Tomcsik and Kurotchkin (1928) to shock guinea pigs passively sensitized with the corresponding antibacterial sera, and Avery and Tillet (1929) secured similar effects with type-specific polysaccharides of pneumococci, including the nitrogen-free Type 3 polysaccharide. Similarly, azodyes, in particular those containing two haptenic groupings to the molecule (made by such devices as a double coupling to resorcinol), and having probably a larger structure owing to aggregation of molecules of the dis-azodye, can cause anaphylaxis directly, without use of protein carriers; this matter has been pursued more recently by Campbell and McCasland (1944), who showed further the inhibiting effect of univalent haptens.

**Reversed Anaphylaxis.** The production of the syndrome of anaphylaxis in a normal animal by injecting first the antigen and then the corresponding antibody has been termed "passive reversed anaphylaxis." This has been attained in several species, as the rabbit, mouse, and even the guinea pig (Opie and Furth, 1926; Schiemann and Mayer, 1926; Kellett, 1935; and Zinsser and Enders, 1936), but commonly requires the use of very large amounts of antibody; reports of shock following practically simultaneous injections of antigen and antibody are discussed in the section on passive transfer. A special instance, described as reversed anaphylaxis, is presented by those animal species in whose tissues Forssman heterophile antigens are present, for instance the guinea pig. If an antiserum produced against some other heterophile antigen is introduced intravenously (such as an antiserum against sheep erythrocytes developed in the Forssman-negative rabbit), the

antibody reacts with the guinea pig's tissues and leads to death with pronounced anaphylactic features; the isolated guinea-pig uterus, however, is said not to respond to such antisera.

**Anaphylactic Shock Elicited by Cells.** Animals that possess antibodies directed against erythrocytes and other cells owing either to active or passive sensitization may exhibit anaphylactic symptoms when the corresponding cells are injected intravenously, but not usually when another route of injection is chosen, nor in tests with isolated organs. The interpretation of these reactions, apart from instances in which the cells simply furnish readily diffusible, soluble antigenic substances or liberate such upon lysis (Friedli, 1925), is questionable: sometimes intravascular agglutination and embolism occludes vessels (Coca, 1909) and gives rise, as do several sorts of particulate matter, to anaphylactoid phenomena (see below), but in other cases truly anaphylactic processes seem to participate (Hyde, 1926; Grove, 1932; Kritschewsky and Friede, 1925). Perhaps the situation is like that obtaining when anaphylaxis tests are made with tobacco mosaic virus, which has a large molecular size: this protein sensitizes, and causes anaphylactic shock upon intravenous injection, but it fails to give Schultz-Dale tests with isolated uterine strips (Seastone, Loring and Chester, 1937).

Like effects are seen with intact bacteria, but evaluation is complicated because of inherent toxicity of the suspensions.

#### ANAPHYLAXIS IN THE DOG

Thus far we have confined our remarks to anaphylaxis as it is observed in the guinea pig. Many other animals show the phenomenon, each species in its own fashion owing in part to anatomic differences in the quantitative distribution of smooth muscle. In the dog, several injections of native protein are usually required to sensitize adequately.

In such animals, injection of the antigen by the intravenous route is followed by restlessness, then vomiting, salivation and diarrhea (at times bloody), and finally profound collapse with loss of muscle tone and slow, often labored respiration; at the same time, the blood pressure and the body temperature both

decrease markedly. Apart from rapidly fatal cases, recovery from the initial shock may be swift, or there may ensue some evidence of improvement and then a second collapse, terminating in death within one to several hours. In the most acute and less protracted instances of anaphylactic death, necropsy discloses chiefly an enormously distended and congested liver (indeed it may contain as much as 60 per cent of the blood); but if shock has lasted for several hours, there is in addition a general congestion of the gastrointestinal tract. The engorgement of the liver has been referred to capillary dilatation and to injury and consequent edema of the liver cells, with a slowing of the circulation; evidence for anaphylactic constriction of the hepatic veins remains unconvincing. Dean and Webb (1924) have carried out careful histologic studies during shock.

As with the guinea pig there is a loss of coagulability of the blood, and similarly there is a decrease in serum complement; a pronounced leukopenia in the peripheral blood reflects the elective retention of polymorphonuclear leukocytes in the lung capillaries, where masses of white cells adhere tenaciously.

During shock, reactions have been shown in various organs *in situ* (Manwaring et al., 1925), and the resemblance to guinea-pig anaphylaxis will be apparent: there is contraction of uterus, intestinal tract (chiefly the colon and the rectum) and urinary bladder: and some degree of bronchoconstriction has been reported as well. Tested alone by perfusion with antigen in Locke's solution, these organs are indeed individually sensitive, but are, excepting for vasoconstriction in the isolated lungs (Manwaring et al., 1923), only trivially so.

The stimulus for the acute reactions which they exhibit in shock comes from the liver. In the course of a remarkable series of investigations, Manwaring showed that exclusion of the liver from the circulation would protect the dog of average sensitivity from exhibiting symptoms of anaphylactic shock upon injection of the specific antigen. Conversely, the sensitized liver itself, when brought into shock while it was joined to the circulation of a normal dog, caused responses even in the normal tissues. The active material has been proven more recently to be histamine, through the



efforts of Dragstedt and his colleagues and of Code (Code, 1939, 1944), and has been considered adequate to account for the vascular reaction. Canine anaphylaxis in highly sensitized dogs, however, can be secured even after extirpation of the liver (Waters et al., 1938).

The dog liver is particularly rich in histamine and gives it off in shock, and at the same time releases large amounts of heparin, influencing thereby the coagulability of the blood.

While the isolated liver perfused with antigen in Locke's solution shows practically no response, Rocha e Silva has found that when perfusion is made with whole blood the liver releases histamine, and participation of leukocytes and platelets in the process is indicated; the latter appear to undergo explosive disruption; the white blood elements themselves, as in the case of the guinea pig and to a much greater degree the rabbit, may contribute some histamine (Katz, 1940). Rocha e Silva et al. (1947) have also shown an activation of plasma protease during anaphylactic shock, and platelet disintegration may be involved here as well; they suggest that it may be the plasma protease which liberates histamine and heparin from liver cells.

The researches of Manwaring point to a more complicated state of affairs in the dog than is present or has so far been recognized in the guinea pig. For example, the organs and tissues of "antianaphylactic" dogs, that is, of individuals themselves not anaphylactic owing to repeated injection of antigen, appear to possess acquired, specific tissue immunity. When the hind quarters of such a dog are connected with the circulation of a dog that is anaphylactic to the same substance and specific shock is induced, the "antianaphylactic" bladder fails to participate in the general anaphylactic reaction; but if "transplantation" has been made in dogs that are anaphylactic towards unrelated substances the bladder will participate in the general shock induced by such antigens.

#### ANAPHYLAXIS IN THE RABBIT

Several preparatory injections of antigen are usually required to sensitize rabbits, and even then systemic shock is not experienced

regularly. The sequence of events upon intravenous injection of antigen differs from both the guinea pig and the dog: there is arrhythmic respiration, then panting, with temporary hyperemia in the ear followed by arteriolar contraction and blanching: the rabbit becomes weak and collapses, gives a few convulsive movements, defecates, and dies suddenly, with head retracted and eyes in exophthalmos. Bronchospasm is absent, but the lung is nonetheless the main organ of shock.

At necropsy, there is found extreme dilatation of the right half of the heart, and engorgement of the inferior vena cava, portal vein and liver; Coca in 1919 showed that the right-sided dilatation of the heart was the direct consequence of an obstruction of the pulmonary arterioles owing to contraction of the surrounding abundant smooth muscle, and he pointed out that death is attributable to secondary heart failure. The blood pressure in the carotid falls greatly, while the pulmonary pressure increases markedly. Coagulation of the blood is somewhat delayed.

In the rabbit it is possible that reaction of antigen and antibody in the blood stream may be more significant than the union of antigen with antibody in intimate relation with the tissues.

There are several lines of evidence pointing in this direction. First, in the actively sensitized rabbit, unlike the guinea pig, there is a significant parallelism between the severity of shock and the concentration of circulating antibody (Jackson, 1935). Secondly, formed blood elements (platelets or leukocytes), which in the rabbit are rich in histamine, release physiologically significant amounts of histamine into the plasma under the stimulus of antigen-antibody interaction, not only upon addition of antigen to fluid blood or to the buffy coat thereof taken from a sensitized rabbit (Katz) but even upon simultaneous addition of antigen and antibody to normal rabbit blood (Dragstedt). The participation of platelets in histamine release has been reviewed by Rocha e Silva (1948). Then again, the refractory state has not been surely achieved in the anaphylactic rabbit, and, conversely, passive sensitization, which is possible with adequately large volumes of serum (Arthus,

1919), may be demonstrated without a preliminary incubation period.

The "stickiness" of leukocytes and the clumping together and clinging of these cells to the vascular endothelium which has been mentioned as occurring in the dog lung may be observed in newly regenerated blood vessels of the rabbit ear (Abell and Schenk, 1938) and has been shown to take place in the sensitized rabbit lung under perfusion with antigen and whole blood; it may be that blood platelets are removed from the blood at the same time. The clumps of leukocytes are even sufficient to obstruct small vessels.

While anaphylaxis in isolated and perfused organs is not well demonstrable, constriction of isolated strips of the pulmonary artery has been shown (Grove, 1932), and anaphylactic constrictions have been shown in the vessels of the perfused rabbit ear (Abell and Schenk, 1938); in the lung there is a resistance to the flow of saline perfusion fluid containing antigen (Friedberger and Seidenberg, 1927).

As with other animals, temporary anaphylactic desensitization seems to be attained by a few subcutaneous injections of small amounts of antigen in the course of three or four hours; this procedure is employed at times in resuming intravenous injections with certain antigens following periods of rest, in the well-known alternation of courses of injections and resting periods so commonly practiced with rabbits for the purpose of securing potent antisera.

The occurrence of striking skin reactions (the Arthus phenomenon) in the anaphylactic rabbit will be mentioned later.

#### ANAPHYLAXIS IN OTHER ANIMALS

Anaphylactic shock is known in other animals, and will be referred to only briefly. Virtually all animals exhibit the phenomenon, but in many there seems to be no dominant "shock organ" such as has been said to be a "characteristic" of anaphylaxis. Undoubtedly we have come to an oversimplified way of looking at the phenomenon by confining our attention principally to the guinea pig, dog, and rabbit. Possibly the train of events in most species is better understood by comparison with the guinea pig that undergoes a protracted death than

with the one whose life is extinguished by early suffocation.

A rough correlation has been pointed out between the ease of establishment of the anaphylactic state and natural sensitivity to histamine (Table 12), but as to mechanism of

TABLE 12. LETHAL TOXICITY OF HISTAMINE BY THE INTRAVENOUS ROUTE\*

	Mg. Kg. BODY WEIGHT
Frog.....	1,700
Mouse.....	250-300
Rat.....	170-500
Monkey.....	50
Dog.....	3
Pigeon.....	1.5
Rabbit.....	0.6-3.0
Guinea pig.....	0.3-0.4

\* Data compiled by R. L. Mayer.

anaphylactic shock only a few studies have appeared. Some species do not become sensitized readily or highly, the rat for instance, but rats may be sensitized by a series of injections and will exhibit a moderate degree of shock when tested within a rather critical period—after a resting period of from 10 to 15 days (Hochwald and Rackemann, 1946a, 1946b). Mice present an unusual situation: while they can be sensitized anaphylactically by several injections (Weiser, Golub and Hamre, 1941), they are highly resistant to intoxication by histamine. Mice can be sensitized passively with rabbit and guinea-pig immune sera and according to Burdon (1946) no incubation period is necessary. McMaster (1941) has employed the ear of the actively sensitized mouse for delicate direct observation of anaphylactic arteriolar and venous spasm following intravenous injection of antigen; no participation of capillaries or lymphatics was found.

Anaphylaxis in the monkey has been studied by Kopeloff and Kopeloff (1939), and by Kinsell, Kopeloff et al. (1941). Sensitization is not easy. Unexpectedly, Schultz-Dale reactions were not secured with uterine strips or intestinal segments of sensitized animals. With horse serum as antigen, the time of reaction to the shocking injection varied from 30 minutes to 24 hours, and, with delayed deaths, edema and hemorrhage of the viscera, par-



ticularly the lungs and intestinal tract, and of the skin were characteristic.

Anaphylaxis in the horse and calf has been restudied by Code and Hester (1939) with sensitizing and shocking procedures patterned on those commonly used with small laboratory animals. Progressive dyspnea, increased peristalsis, and sweating were seen in two horses, and respiratory difficulty appeared to be the cause of death in one horse, which died in 8 minutes. In horses studied by Gerlach (1922) and Ritzenthaler (1924), urticarial responses were prominent, and there was marked edema of the legs. It may be mentioned that horses exhibit dyspnea very commonly during injections with bacterial vaccines (pneumococci), and may show prompt accumulation of fluid in the lungs.

Birds may be sensitized, and in particular several studies have been carried out in the pigeon; the anaphylactic contractility of the isolated circular muscle of the pigeon's crop has been suggested as a laboratory tool.

#### ANAPHYLAXIS IN MAN

Systemic reactions apparently identical with anaphylactic shock of the lower animals have been encountered in man, often associated with urticaria and edema as in anaphylaxis seen in the horse. Bronchiolar constriction is not invariable and there is no one dominant "shock organ"; it has been stated that "anaphylaxis in the human being may resemble any animal type or a combination of them." The literature has been reviewed by Ratner (1943). Fortunately such accidents are rarely fatal, and most of the fatal and grave incidents occur upon administration of horse serum to people having a pre-existing clinical sensitivity to horse dander, that is, "horse asthmatics" (Ratner and Gruehl, 1929). Here, postmortem examination is apt to reveal distended lungs, splanchnic dilatation, and at times engorgement of the liver. The hypertrophy of bronchial musculature frequently developing in such subjects has been interpreted as rendering them particularly liable to respiratory symptoms and has been likened to the condition normally

existing in guinea pig lung. The tissue changes, the presence of passive transfer antibodies, and the type of response are like those occurring in pollen hay fever, where similar accidents are experienced upon employing an overdose of pollen extract. Kallós and Kallós-Deffner (1937) found evidence of enlarged and emphysematous lungs in autopsy records of patients dying in asthmatic attacks, and they pointed out that "the special histologic character of the alterations in *Asthma bronchiale* rests on a peculiar allergic reaction of the bronchial wall and of the lung tissue" (translation).

Among individuals who have become sensitized to foreign serum because of prior administration, those who have become so hypersensitive that they give the "immediate-type" of reaction upon skin testing are most prone to systemic accidents. The most severe response comprises collapse, fall of blood pressure, tachycardia, dyspnea of the asthmatic type, suffusion of the face, urticaria with giant wheals, sometimes marked edema of the entire body (Longcope and Winkenwerder, 1941); in fatal cases, death may occur within a few minutes or as late as 24 hours. Odd and unpredictable, but comprehensible, serum accidents may occur (Fawcett and Ryle, 1923). Spaced, intrathecal injections of antimeningococcal horse serum in cases of meningitis have resulted in ostensibly anaphylactic symptoms.

Anaphylaxis has been noted following repeated injections of tetanus toxoid and of a prophylactic preparation of alum-precipitated diphtheria toxoid and pertussis antigen (Werne and Garrow, 1946); in the latter instance, the role played by hereditary factors seems obvious. With this preparation, identical twins died in delayed shock, 16 and 24 hours respectively after the second injection. Both children showed histopathologic evidence of acute vascular injury and edema, constricted arteries, and tissue eosinophilia.

## ANAPHYLACTOID REACTIONS

There are many materials which, upon injection, cause normal animals to exhibit in variable degree the gross symptoms of anaphylactic shock. Since an antigen-antibody interaction is not the primary trigger mechanism, such reactions are termed anaphylactoid. Naturally enough, these reactions have been studied eagerly in the belief that they would help to explain the basic mechanism in anaphylactic shock.

The most precise replica of anaphylactic shock is secured by the intravenous injection of bacteriologic "peptone," the effect being independent of any histamine present. The guinea pig succumbing to "peptone shock" shows smooth muscle contractions and the characteristic exitus with fully inflated lungs. The mechanism appears to involve, among other factors, liberation of histamine from the tissues (Dragstedt), which produces the shock: the quantity so released is of the order of magnitude of that set free in anaphylactic shock, and acts chiefly at the site of liberation. Likewise, crystalline trypsin injected intravenously into the intact animal or used for organ perfusion liberates histamine and thereby causes shock (Rocha e Silva and others), and the same holds for perfusion of guinea pig lung by snake venoms and staphylococcal toxin (Feldberg, 1937; 1941).

In addition to the above procedures, normal serum from an animal can be treated so as to produce anaphylactoid reactions upon introduction into normal animals of the same species: it becomes toxic after incubation with various substances—kaolin, barium sulfate, starch, agar, and the like, and so does whole blood merely by withdrawal and reinjection in the "preclot stage" (Novy, DeKruif, et al., 1917). As a class, these preparations have been designated "toxified sera" or "serotoxins." The effect is sometimes attributed to removal, by absorption, of antiproteases which usually hold in check the action of serum enzymes on serum proteins, but the mechanism has not been elucidated.

Hanzlik and Karsner in studying responses to a wide variety of agents injected intravenously—typhoid vaccine, bile, agar, gum acacia, starch, arsphenamine, etc., found that while gross features of anaphylaxis were reproduced in more or less detail, the death, as observed in the guinea pig, was apt to be

of circulatory rather than respiratory origin and to involve occlusion of vessels by white cells or fibrin.

## THE MECHANISM OF ANAPHYLAXIS

The mechanism by which anaphylactic shock is brought about is by no means simple or fully understood, despite the many efforts directed to this end. Any adequate theory must of course account not alone for acute death but also for instances of protracted shock and for late deaths. From many ways of experimental approach, however, a broad pattern is emerging. According to current concepts, the important element is damage to the tissues, particularly the vascular endothelium, either by antigen-antibody interaction or by non-specific materials such as "peptone" or "serotoxins"; subsequent events are determined largely by materials leaving the damaged tissues and by the very fact of tissue damage itself.

As the terminology still prominent in the literature is based on prior concepts, it may be useful to present very briefly some preceding lines of reasoning. (1) Whereas one of the first theories held that anaphylaxis depended upon a reaction between antigen and sessile antibodies ("cellular hypothesis"), this theory was eclipsed when experimental evidence seemed to suggest that a toxic substance (Friedberger's "anaphylatoxin") was liberated in the blood stream and was directly responsible for the anaphylactic symptoms ("humoral hypothesis"); the anaphylatoxin supposedly arose from the antigen as a result of proteolysis following its reaction with antibody (and complement). Actually, Friedemann (1909) was able to show that upon incubation a mixture of antiserum and specific antigen became capable of producing either anaphylactoid or anaphylactic responses in normal animals, and indeed in several animals there is said to be no mandatory "latent period" following passive transfer, that is, injection of antigen immediately after injection of antibody-rich serum results in anaphylaxis (see *Passive Transfer*, p. 116). Also, an *in vitro* model of artificial anaphylatoxin was deduced from the toxicity of "cleavage



products" secured from bacillary bodies by action of alkali (Vaughan and coworkers, 1907). (2) But it was obviously doubtful whether the small amount of antigen needed to produce prompt and fatal shock in sensitized guinea pigs could be adequate as substrate protein for the production of "anaphylatoxin." Actually, this idea was finally eliminated in 1928 by the finding that typical shock could follow the injection of nonprotein substances (microbial polysaccharides) into sensitized guinea pigs (page 118). The discovery of the anaphylactoid reactions produced by the various "serotoxins" and by blood in the preclot stage (Novy, DeKruif, et al., 1917) then led to the suggestion that the substrate for anaphylatoxin formation could be the host's proteins: according to one view, the antigen-antibody complex would function as an agent for absorbing antiproteases so that plasma proteins, attacked by serum enzymes, would produce "anaphylatoxin" (Jobling, Petersen and Eggstein, 1914; Bronfenbrenner, 1915). (3) In 1913, Dale demonstrated that the excised uterine tissue of sensitized guinea pigs reacts to contact with specific antigen, and thereby confirmed the less conclusive report of Schultz (1910) with intestinal segments. The uterine tissue reacted even after most of the animal's serum had been removed by perfusion. These experiments appeared to be crucial: they focussed attention back to the cell and suggested the participation of "sessile" antibody; obviously any serum protein present was insufficient for the formation of "serotoxins" and other such "anaphylatoxins."

The present concept holds that the reaction between antigen and cell-fixed antibody results in damage to the tissues. Various suggestions have been advanced in attempting to explain how this serologic reaction could cause damage to the cells. One idea is that tissue injury may result from proteolysis, and indeed there is evidence for the activation or freeing of a proteolytic enzyme during anaphylactic and anaphylactoid shock (Ungar, 1947; Rocha e Silva et al., 1947), either indirectly by interference with antiproteases that normally inhibit the enzyme function or by activation of pro-enzyme. After the combination of antigen with antibody, the tissues then lib-

erate various substances, some of which participate in the production of the anaphylactic syndrome: histamine (previously held in an inactive form, attached perhaps within lipoprotein films), heparin, adenosine derivatives, acetylcholine, potassium ions and possibly still other pharmacologically active entities, such as the special "slow-reacting substance" detected by Kellaway and Trethewie (1940) in perfusing the lungs of sensitized guinea pigs, and other substances as yet only of hypothetical interest.

Thus certain enzymes act on lipins to produce pharmacologically active "lysocithin" (a mixture of lysolecithin and lysocephalin), but it is not known whether an analogous event—intracellular splitting of tissue lipins—occurs in anaphylaxis. These studies have been conducted with enzymes present in snake venoms, and with lecithin and egg yolk as a prototype of tissue lipins (Feldberg, 1941).

One may speculate whether tissue injury follows or precedes the appearance of increased proteolytic activity. Proteolysis is reported to occur widely during shock, even in organs that are not able to contribute significant amounts of histamine. Any proteoses and peptones formed intracellularly as a result of proteolysis might well be physiologically active (Feldberg, 1941).

The participation of liberated heparin in reducing the coagulability of the blood has already been noted.

A decision as to the role of the various substances in shock is obviously difficult to reach, for minute amounts liberated locally within sensitized tissues may represent effective concentrations, but may escape detection in the circulation. Indeed, the occurrence of several simultaneous reactions with different mechanisms has been suggested.

#### THE ROLE OF HISTAMINE

The extent to which histamine has been implicated in the production of anaphylac-

tic shock is, as already indicated, not inconsiderable (Code, 1944; Dragstedt, 1945); in guinea pig, rabbit and dog, it may well alone be responsible for the acute form of death. It was noted early that histamine imitates fairly well, upon intravenous injection into normal animals, the characteristic species-peculiarities of anaphylactic shock and the mode of death—constriction of pulmonary vessels of the rabbit and right-sided heart failure, constriction of guinea pig bronchioles, and vascular effects in the dog. Increased amounts are clearly detectable during shock, and the question is whether the histamine released is sufficient to account for the acute shock. This is not to be decided readily. The values found are small, but this would be expected because at a given time only a part of the total histamine would remain in the plasma. Obviously it is not possible to assess the concentrations attained locally in histamine-sensitive tissues. According to Dragstedt and Mead (1936), the intravenous injection of histamine into dogs in amounts providing plasma concentrations similar to those encountered in shock would produce manifest and characteristic histamine effects.

The sources of histamine liberation differ according to the species; the histamine content of the various organs does not foretell the amount set free in shock. In the guinea pig, it arises in appreciable quantities from many tissues—aorta, lungs, uterus, seminal vesicles, *inter alia*, and, moreover, by simple contact of the isolated perfused sensitive tissues with antigen at body temperature (Bartosch et al., 1932, 1933; Schild, 1939). In the dog, the chief contribution comes from the liver, possibly with participation of white blood elements. In the rabbit, the white cells or the blood platelets are rich sources of histamine, and in shock histamine is liberated into the plasma; but this circumstance was not appreciated for long, since white cells leave the circulation during shock and consequently determinations of histamine levels on whole blood showed a histamine deficit during anaphylaxis. However, as Feldberg suggests, the main release of histamine may

occur in juxtaposition to the pulmonary arteries.

There are, however, observations pointing against histamine as the sole mediator of shock, unless one would attribute quite different effects to "H-substance" (histamine) released in the cells and histamine applied from without. The guinea-pig uterus, poisoned by histamine so that it responds to further histamine solely by relaxation, nevertheless contracts to addition of specific antigen and to certain agents which produce anaphylactoid responses—peptone (Schild) and snake venoms (Kellaway, 1938). With mice, the amount of histamine existing preformed in the body is said to be only one-tenth of the amount necessary for fatal histamine shock by the intravenous route, and the antihistaminic drugs not only fail to afford to the mouse typical protection against anaphylactic shock but act synergistically with histamine (Mayer and Brousseau, 1946). The rat, likewise, is relatively insensitive to histamine, and—an observation which should be confirmed because of its importance—it is said that the rat uterus may be caused to contract in balanced salt solution by the presence in the same bath of sensitized guinea-pig lung and corresponding antigen (Campbell and Nicoll, 1940), all the histamine contributable by the lung being far below the physiologically effective amount for rat tissues. Dogs may recover from the initial symptoms of anaphylaxis, to succumb from later profound shock when the histamine levels in the blood are normal.

A critical review, with consideration of further evidence, is given by Rose (1947).

#### ACETYLCHOLINE

A few workers have considered a "cholinergic" rather than a histaminic causation of anaphylactic shock (Nakamura, 1941; Chigira, 1941; Went, 1939; Daniélopou, 1946), but the evidence for a major role of acetylcholine in shock is still unconvincing. The



addition of acetylcholine to isolated smooth muscle causes typical contraction, and the intravenous injection of large amounts of acetylcholine, performed rapidly to forestall simultaneous enzymatic inactivation in the tissues, duplicates the symptoms of anaphylaxis by producing excitatory symptoms in organs that are stimulated by the parasympathetics, not by the sympathetics (bronchi, digestive tract, uterus, bladder) and inhibitory symptoms in organs that are stimulated by the sympathetics, not by the parasympathetics (chiefly the heart, thus leading to lowering of blood pressure). According to this theory, when there occurs an imbalance between the normal formation of acetylcholine and destruction of it by cholinesterase, in the direction of an increase in acetylcholine, shock is produced. The cells are considered to liberate acetylcholine directly, without intervention of nervous impulses, by reason of antigen-antibody interaction, or direct stimulation of cells as by agar. Unlike other tissues, the sensitized guinea-pig heart gives off choline and acetylcholine during shock (Went, 1936; Farber et al., 1944).

#### ALLERGIC INFLAMMATION: EARLY RESPONSES

When relatively small amounts of antigen or allergen are introduced into the tissues of a sensitive individual, contact is localized, and the rate of absorption or dissemination is retarded by tissue barriers and by existing antibody mechanisms. The primary response of the tissues concerned is a local inflammation, initiated and for a time kept stimulated by the allergic mechanism. This "allergic inflammation" is far more violent than the mild, transient inflammations that are detectable histologically when the same material is injected into non-sensitive individuals. As stated before, these specific reactions fall into two broad categories, not always clearly separable—the early and the "delayed" (tuberculin-type) reactions. We shall reserve discussion of the latter type and consider now the responses that, like anaphylaxis, become evident

shortly after introduction of the corresponding allergen or antigen.

The occurrence of the early type of local reaction, both in animals and in man, often coincides with the presence in the serum of antibodies that are capable of transferring corresponding sensitivities to normal subjects. This has been shown following blood transfusions or bulk injections of serum, procedures which result in a sensitivity of the entire skin, and also following intracutaneous deposition of serum, a method which sensitizes the skin in a local area only. Some degree of sensitivity then persists for a variable period, according to the species and the concentration (or type) of antibody in the serum employed. Such a site will react when antigen is brought to it (by local injection or by conveyance in tissue fluids), and it is then desensitized. The reactions in such prepared sites are of the evanescent type, and reproduce the sensitivity of the serum donor.

In thus linking together all instances of transfer of sensitiveness by serum, and in seeing therein a general principle of immunology, we must make it clear at the outset that questions regarding the nature of the antibodies concerned, and which subjects are capable of producing them, are not to be resolved simply. Also, it must be reiterated that some forms of food hypersensitiveness in man—properly to be regarded as reactions of early type—can occur without evidence for circulating antibody and without positive cutaneous reactivity to the offending allergen ("familial nonreaginic food allergy" of Coca).

Because of their manifest relation to antibodies, the local reactions in anaphylactically sensitized animals claim first attention. The special conditions requisite for the demonstration of anaphylactic shock—adequate sensitization, a proper interval following the sensitizing contacts, and finally the introduction of specific antigen in adequate amount—obtain in the

demonstration of tissue reactions. As Opie has said (1936), both anaphylactic shock and the variety of allergic inflammation that is produced by foreign proteins "are antigen-antibody reactions, subject to passive transfer and to desensitization by excess of antigen."

When antigen is deposited in the skin or subcutaneous tissues of anaphylactically sensitized animals, local allergic inflammatory responses start almost at once, with hyperemia and edema, these reactions being either frankly evanescent or more persisting (the so-called "Arthus type," to be described below). It is not clear what underlies the difference between these varieties of expression, but some have attributed it solely to relative concentration of antibody (cf. Rich, 1944). The guinea pig, for example, gradually develops a more prolonged response with continued sensitization.

The more persisting variety of allergic inflammation, the Arthus reaction, will be discussed first. Among the laboratory animals it comes to fullest expression in the rabbit, but it occurs, usually after many preparatory injections, in other species, including horse (Gerlach, 1922), monkey (Kopeloff and Kopeloff, 1939), guinea pig (Arthus; Cannon and Marshall, 1941), and man; it cannot be induced readily in the rat. The well-developed reactions of the rabbit may perhaps be a consequence both of the high antibody titers encountered in this species and of the content of available histamine liberated by white blood cells (or platelets) in the presence of antigen and antibody (Katz, Dragstedt).

#### THE ARTHUS REACTION

Historically, hypersensitivity in the rabbit was first observed as a local, not a systemic reaction (Arthus, 1903). Rabbits repeatedly injected beneath the skin with horse serum, at appropriate intervals, came

to respond with progressively more intense reactions at the site of each succeeding injection. The earliest response was a transient local swelling, the ultimate response was hyperemia and edema followed by hemorrhage and intense induration requiring a day or more to develop and progressing to deep necrosis and sloughing. The reaction became known as the "Arthus phenomenon." Since reactions are observed more clearly and necrosis is more readily obtained by the intracutaneous injection of antigen, the original procedure of Arthus is frequently replaced by injections into the skin.

In addition to the cutaneous and subcutaneous reactions demonstrable in the sensitized rabbit, injection of antigen into many other sites results in marked inflammatory response—stomach submucosa, kidney, liver, brain, lung, testicle, joints, ligatured blood vessels (Seegal, 1935; Opie, 1936; Cannon, Walsh and Marshall, 1941)—and striking inflammation of the pericardium and myocardium have followed instillations of antigen into the pericardial cavity (Seegal, 1935).

The intensity of the reaction varies with the antibody level in the serum (Culbertson, 1935). The capacity to react, as with anaphylactic transfer in the guinea pig, may be transferred to normal rabbits by injection of the serum from actively sensitized rabbits (Nicolle; Opie, 1924a): if such serum is given in quantity intraperitoneally or intravenously, a reaction may be elicited anywhere on the entire skin area of the recipient rabbit, whereas if the antibody is injected into the skin, a local reaction will occur at that site if very shortly thereafter antigen is given either locally or intravenously.

The amount of antibody required for transferred Arthus reactions of minimal intensity has been measured by Fischel and Kabat (Kabat, 1947) and is found to be as little as 0.15 mg. antibody protein when deposited intracutaneously (cf. Culbertson, 1935; Cannon



and Marshall, 1941). Such transferred Arthus reactions, performed on the skin of the back, develop slowly, but it is interesting to note that the same procedure carried out on the rabbit's ear—a tissue in which direct Arthus reactions are not encountered (Gerlach, 1923)—results in immediate-type, evanescent responses (Ramsdell, 1928).

The Arthus phenomenon is usually considered as a "local" anaphylactic response of the rabbit, despite the number of hours required for full development and the relative persistence of the tissue change. The reaction actually is initiated promptly, but some hours may be necessary for the development of a macroscopically visible reaction. Microscopically, even within an hour after subcutaneous injection of antigen (Gerlach, 1923), the site of deposition may exhibit evidence of marked alterations in capillary permeability—intense swelling of connective tissue fibrils with compression of the blood vessels, a surrounding massive edema and immobilization of leukocytes. The process is at maximal histologic expression at 24 hours, when there is necrosis of the arteriolar vessel walls and consequent hemorrhage, fibrinoid degeneration in the connective tissue, and adventitial inflammation and edema. The primary vascular damage would accordingly account for edema, thrombosis of vessels and eventual necrosis and sloughing. Some observations appear to indicate that the tissue cells at large are not hypersensitive, for instance the lack of injury when cells in tissue culture are in contact with the specific antigen, or when antigen is injected into the relatively avascular cornea (Aronson, 1933; Rich and Follis, 1940).

The Arthus reaction may be elicited repeatedly on an animal, but if desensitization is undertaken by means of a massive dose of antigen given beneath the skin, subsequent reactions are diminished or abolished (Opie, 1924b; Culbertson, 1935; Cannon and Marshall, 1941): the anaphylactic rabbit is notoriously difficult to desensitize.

Lesions of the Arthus type have been reported in man, usually upon injection of foreign serum and in instances in which a prior administration has given some urticarial signs indicative of an existing sensitivity (Kojis, 1942; Ratner, 1943).

Local reactions of this sort, known to Arthus in 1903, have been reproduced unintentionally in children by giving them subcutaneous injections of antitoxic horse serum at three-week intervals (Lucas and Gay, 1909): between the second and sixth injections there appeared, in addition to scattered cases of generalized urticaria, the characteristic local changes—edema, tenderness, erythema, and marked, persistent induration or eventual necrosis. At times when therapeutic injections of immune horse serum are given to severely ill, sensitive patients, the reactions are extraordinarily extensive and may show black, dry eschars over large areas, as noted by Gatewood and Baldrige (1927) and Tumpeer et al. (1931), or may even lead to extensive slough of the skin and subcutaneous tissue (Kohn, McCabe and Brem, 1938). Whether there is some auxiliary role attributable to concomitant bacterial disease is unknown. Passive transfer of local serum reactivity in man has been reported by Kojis (1942).

#### EVANESCENT ALLERGIC INFLAMMATION

Local reactions to foreign proteins are more transient in man and other animals, as a rule, than they are in the highly sensitized rabbit. Nevertheless, from histologic studies of such local reactions, Gerlach (1923) concluded that the tissue alterations are analogous to, and differ only in degree from, the Arthus reaction in the rabbit (cf. Laporte, 1934). Depending upon the degree of sensitiveness and the availability of antigen, the subject will react within 15 to 90 minutes at the site of intracutaneous injection and develop erythema and edema of variable grade. In the guinea pig, for example, at the level of sensitivity frequently studied, incipient swelling and hyperemia develop within 5 to 15 minutes following an intracutaneous test, the reaction increases for 30 minutes up to a few hours, when it is softly edematous and may measure 2 or 3 cm. in diameter, and then it fades gradually and disappears within 2 to 4 hours (Zinsser, 1921). After further sensitizing injections, the reaction is some-

what more pronounced in its early stages and is apt to be less fleeting, and it may continue to increase for perhaps 18 hours (Dienes and Schoenheit, 1927; Kellett, 1930). But with intense sensitization, frank Arthus reactions can be obtained.

By the transfer of antibody-rich serum from rabbits or guinea pigs to normal guinea pigs, a cutaneous reactivity of the evanescent type is temporarily established as well as an anaphylactic state (Zinsser, 1921; Dienes and Schoenheit, 1927; Ramsdell, 1928; Kellett, 1930; Chase, 1947). Either a general or a local reactivity of the skin may be induced at will; in the latter case the anaphylactic sensitization can escape detection.

Up to this point we have been considering chiefly the local inflammatory reactions which occur in subjects known to be anaphylactically sensitive. Apparently fundamentally similar to these responses, and likewise generally transferable by means of serum, are the evanescent cutaneous reactions that occur in man. In the sensitive human being, an exceedingly interesting situation has come to light, having to do with the formation of several varieties of antibody, and with the influence of hereditary constitution in determining the occurrence of allergic sensitization. The intricacies were such as to suggest for long that man stood, immunologically speaking, apart from other animal life, but quite recently evidence seems to be accumulating for the presence of somewhat similar varieties of antibodies in the lower animals. As yet, no final appraisal can be made.

The early type of response is far more pronounced in man than in the lower animals, a situation referable to such species differences as a form of antibody that is retained longer in the skin, a marked cutaneous reactivity to histamine, and the possession of a rich superficial lymphatic network. The characteristic reaction in the skin is the "wheal and flare," that is, a

sharply circumscribed, elevated, blanched area sometimes showing pseudopodial extensions, and a surrounding, erythematous flare; the entire area involved may vary from 3 to 10 or more centimeters in diameter (Plate 1). The reaction usually begins to fade within 15 to 60 minutes, after which the skin may regain its normal appearance.

The successive events—primary erythema, spreading flush or flare and central whealing—were termed the "triple response" by Sir Thomas Lewis (1927), and he brought evidence that all three could be referred to the actions of a diffusible "H-substance" and perhaps other active materials, liberated locally in the wake of cellular injury (owing to antigen-antibody interaction or to direct injury by a variety of stimuli). The relation of this concept to current theories on the production of anaphylaxis will be apparent. This diffusible agent, now generally accepted as histamine, was considered to initiate the following successive phases: (1) local vasodilatation of capillaries (initial erythema), (2) a local axon reflex resulting in widespread arteriolar dilatation (flare) and finally (3) a further action on the endothelium of the minute vessels with an increase in permeability and development of pronounced and circumscribed edema (whealing). This interpretation has not found acceptance everywhere (Ratner, 1943), but in any event an intracutaneous injection of histamine into the human skin reproduces the triple response pictured above.

So far as direct evidence for the participation of histamine in allergic conditions of man is concerned, it may be said that the appearance of increased amounts of histamine has been sought repeatedly, but chiefly with equivocal results (Rose, 1947); transitory increases have been detected, however, in instances of physical allergies (photosensitivity, allergy to cold, dermatographia). The failure to obtain more concrete evidence is not unexpected in view of the rapidity with which histamine can be removed from the blood (Rose, 1940), especially when only small amounts, producing local symptoms, are involved. Obviously the situation is not yet clarified, and it has been suggested that in certain patients some substance other than histamine (acetylcholine?) may be involved.



## SERUM DISEASE

Soon after the discovery of diphtheria antitoxin, the illness known as "serum sickness" was observed in a small proportion of the patients treated by therapeutic injections of antitoxic horse serum; later, with the employment of horse antiserum in still larger doses, e.g., antistreptococcal, antimeningococcal and antipneumococcal sera, the case incidence increased markedly. A masterly and comprehensive monograph was written by von Pirquet and Schick in 1905; the theoretical conclusions at which they then arrived have been largely confirmed. Following an initial injection of foreign serum there is usually an incubation period of 6 to 10 days, closely similar to the time required for development of the anaphylactic state in the guinea pig. At that time, as a consequence of the interaction between specific antibodies then present and the remaining traces of foreign proteins, there occur a generalized swelling of lymph nodes, an urticarial or erythematous eruption with itching, and often an edema of the eyelids, face, and ankles; in severe cases, arthralgia and fever follow the eruption.

These and other symptoms are described in detail by Longcope and Winkenwerder (1941) and by Ratner (1943). The disease may be transient or it may last up to 2 weeks; the usual period is slightly over 2 days. With crude horse serum there may be one or more recurrences of the eruption, attributable to the fact that antibody formation to the various serum proteins may be established at different times.

If the antibody-producing mechanism has been established by some previous but not too recent injection, a new administration of horse serum will bring on the same train of events after a shorter interval, 3 to 5 days ("accelerated reaction"), paralleling the "recall" phenomenon observed when animals are restimulated with antigen after a rest period.

Reinjection at a time when the sensitivity is sufficiently high may cause local immediate reactions, within 15 minutes to an hour, and

also general immediate reactions, occurring within some minutes to 12 hours; generalized immediate reactions are often severe and may occasionally prove fatal. Testing for immediate-type sensitivity is generally practiced by cautious intracutaneous injection prior to therapeutic administration of antisera of animal origin.

It should be mentioned that clinical manifestations closely resembling "serum disease" can occur as a consequence of drug therapy, for instance with the sulfonamides and the arsphenamines (Longcope, 1943).

The recognition of human serum sickness as an allergy was accompanied by the discovery in patients' sera of antibodies giving precipitation in vitro with horse serum and anaphylactic sensitization in the guinea pig (Longcope and Rackemann, 1918; Tuft and Ramsdell, 1929), and like findings appeared in certain allergies to common foods (Schloss, 1912). Even von Pirquet and Schick, however, could not relate the cutaneous hypersensitivity towards horse serum with precipitating antibody in the circulation, a circumstance not always remembered later. Nevertheless, the role played by antigen-antibody interaction in serum disease was indisputable, and this mechanism evidently had a bearing on the accompanying cutaneous effects.

A still sharper separation between the cutaneous reactivity demonstrable by skin testing and the possession of ordinary antibodies was evident in an entirely different group of allergic individuals, namely those who had acquired clinical allergies such as hay fever and asthma to pollens, and some of those with food sensitivities: their sera showed no precipitate with extracts of the offending materials, and passive anaphylactic or cutaneous sensitization of the guinea pig, although experienced now and then (de Besche, 1923; Ramsdell, 1930) was rare. Finally, however, antibodies of a special sort, differing from the classic antibodies, were detected (Prausnitz and Kuestner, 1921), and these appear to occur

along with other antibodies in patients with serum sickness.\*

#### THE PRAUSNITZ-KUESTNER (P-K) REACTION

The technic of Prausnitz and Kuestner has since come to be a powerful tool in allergy. Prausnitz received in his skin a small amount of serum taken from Kuestner, who was markedly sensitive to fish, and then, 24 hours later, an extract of fish was injected into the site so prepared. A striking local reaction developed in the course of some minutes and rapidly faded. This technic was then extended, largely through the detailed studies of Coca and Grove (1925), and was found to be applicable in many clinical allergies; such a reaction is illustrated in Plate 1. (In certain cases of exquisite hypersensitivity, diagnosis of the responsible allergen has been made preferentially by testing sites on a normal individual passively sensitized with patient's serum.) Whealing occurs at a sensitized site not only when the allergen is introduced locally but also when it is injected into remote tissues (Lippard and Schmidt, 1937) or into the blood stream, or is absorbed following ingestion or inhalation (Walzer, 1942). One may, alternatively and at the cost of working with submaximal reactions, inject allergen and reagin together into normal human skin.

These antibodies are termed "reagins" or, less precisely, "skin sensitizing" antibodies; they are not to be confused with "Wassermann reagin," which has for long been a designation for the syphilitic antibody.

The reagins can sensitize passively not only human skin but the nasal and ophthalmic mucous membranes and mucous membranes of the intestinal tract; in studies on rhesus monkeys, human reagins have been shown to

sensitize the stomach (submucosa) and gall-bladder (lamina propria) as well. The reactions induced in such prepared sites consisted of conspicuous edema, development of pallor followed by hyperemia, and hypersecretion. In the passively sensitized monkey, as in the food-sensitive human being, administration of allergen in the diet resulted in pronounced gastric retention owing in part to occluding edema near the pylorus; in the bowel, there was either spasm or dilatation. For a summary of these studies, the reader is referred to Walzer (1941).

Reagins are usually encountered in association with "spontaneous" allergic states such as seasonal coryza and asthma, and some kinds of eczema. (In urticaria and angioneurotic edema reagins are hardly to be found.) It was realized early that diseases of this sort showed familial distribution; data to this end were assembled by Cooke and Vander Veer in 1916 and by Spain and Cooke (1924). Their evidence that the age of onset varies according to the probable degree of inheritance—unilateral or bilateral—has not been accepted universally (Ratner et al., 1941). Despite earlier interpretations, the inheritable property was a susceptibility for sensitization only, since the allergens concerned and the type of clinical manifestations varied among sibs and between children and their parents. In some cases it is not possible to trace a previous contact, especially in young babies; it is possible that sensitization can occur through traces of substances provided by the maternal diet. The opportunities for undisclosed sensitization naturally increase with age.

There is, then, a decided influence of heredity on the capacity to form reagins under natural types of exposure to allergenic materials. Obviously, it cannot be known whether the "control" exerted by inherited constitutional make-up is absolute or relative (cf. Urbach and Gottlieb, 1946). Almost always, but not invariably, the possession of reagins coexists with the presence of a corresponding clinical disorder.

\* It may be mentioned that the antibodies present in serum sickness can be demonstrated advantageously by reversing the sequence of injections used in the Prausnitz-Kuestner technic. The reader is referred to Voss, 1938; Wright and Hopkins, 1941; Karelitz and Glorig, 1943.



der. The location of the principal reactive sites ("shock tissue") determines the particular disease, not the mere presence of reagins in the circulation. By adequate artificial immunization, however, reagins have sometimes been produced in the populace at large, for example with extracts of *Ascaris* (Rackemann and Stevens, 1927; Davidson, Baron and Walzer, 1947) and of *Trichina* (Baron and Brunner, 1942), and with horse serum. The development of reagins or the reagin-type of skin reactivity in a sample of the population has been observed several times under conditions of natural exposure, e.g., to dead sewage flies (*Psychoda*) (Ordman, 1946), and to castor bean pomace (Figley and Elrod, 1928), but the relation to inherited constitution is undetermined.

#### REAGINS AND THERMOSTABLE ANTIBODIES

The antibodies demonstrated by Prausnitz and Kuestner, while apparently belonging in the gamma globulin fraction of serum like most other antibodies (Newell et al., 1939), have certain special properties. (1) They are unduly heat-labile, losing their tissue sensitizing function when held at 56° C. for between one-half hour and eight hours, or 60° C. for one-half to one hour. There is no critical temperature, and the degree of heating required for full inactivation varies from one serum to another (Schmidt and Lippard, 1937). (2) Another distinguishing characteristic is the property of sensitizing and remaining fixed in normal human skin (and probably other tissues) for long periods of time, up to 45 days (Lippard and Schmidt, 1937), but at any time a reaction induced by allergen will "discharge" the site. (3) Reagins do not appear to pass the placental barrier. (4) The reagins found in natural cases of hypersensitivity do not precipitate the allergen (and indeed would not be likely to do so unless antibody were sufficiently concentrated). But apparently in special cases

of induced hypersensitivity reagins may appear in admixture with other, precipitating antibodies (Loveless, 1947b). (5) Human reaginic sera are seldom found to render guinea pigs anaphylactic to the allergen, and it could be assumed that occasional positive effects are due to traces of precipitating antibody present at the same time.

The statement is often made that reagins cannot "neutralize" allergen, because of the observation that a mixture of allergen and reagin usually induces a reaction upon injection into the skin of the allergic person. But apparently evidence for neutralization may be found when careful attention is given to the ratio of allergen and antibody in the mixture (Schmidt and Lippard, 1937; Eagle and Loveless, 1947). Likewise suggesting direct in vitro interaction between reagins and corresponding antigens (egg white, milk) are reports that complement fixation can be shown with some samples of reaginic sera when tested with unusually high dilutions of antigen (György, Moro and Witebsky, 1930; Woring, 1931; Lippard, 1939), but the relation of this effect to reagin is not clear. There is another, recent report of the participation of reagins in in vitro reactions, namely, that anti-ovalbumin human reagin combines with reacting mixtures of ovalbumin and precipitating (rabbit) antibody (Miller and Campbell, 1947).

In carrying out the accepted procedures devised to "desensitize" allergic individuals, namely, repeated injection with tiny amounts of their allergens, it came to light (Cooke et al., 1935; Loveless, 1940) that the ensuing clinical improvement is not related to a diminution in the amount of circulating reagins, and it was discovered that the course of injections was actually giving rise to another, thermostable antibody, that is, to one that fails to sensitize the skin and withstands temperatures deleterious to reagins. Even normal individuals under the same treatment give rise to the same antibody, but they produce no reagin (Cooke, Loveless and Stull, 1937; Loveless, 1942).

The thermostable antibody combines

readily and specifically with the allergenic material, thereby "neutralizing" the latter so that a mixture of the two will fail to produce a positive skin test on a sensitive individual (Loveless, 1940). It has, therefore, been termed the "blocking" or "inhibiting" or "neutralizing" antibody, probably only temporary designations. The thermostable antibodies readily leave the skin after injection therein, in this respect resembling rabbit-precipitating serum deposited in rabbit skin (Freund, 1929), and pass the human placental barrier (Sherman et al., 1940). Consequently, it is only a matter of hours, following deposition in the skin, when there remains a concentration of thermostable antibody sufficiently high to be recognizable. Although mixtures of allergen and blocking antibody do not give discernible precipitate, the presence of thermostable antibody seems to decrease the volume of precipitate given by mixtures of allergen and precipitating (rabbit) antibody (Hampton, Johnson, Alexander, and Wilson, 1943).

Quite naturally, development of this second antibody, adjusted like the reagin to the same allergenic material, was considered to be responsible for the clinical improvement and to be the aim of treatment. Logical as the thought is, evidence for this view is incomplete.

It may be mentioned that coexisting reagin and thermostable antibody have been encountered in cases of insulin sensitivity, the thermostable antibody apparently being responsible for periods of "insulin fastness" (Lowell, 1944, 1947; Loveless, 1946).

The existence of a pair of different antibodies both having similar serologic specificity is exemplified not only by reagin and thermostable "blocking" antibody and (in serum sickness) apparently by reagin and precipitating antibody: in the blood typing laboratories, both Rh agglutinins and Rh "blocking" antibodies are encountered among human sera, having the same specificity but differing from one another in heat stability, in penetration through the placenta, and in

capacity to agglutinate Rh erythrocytes in salt solution; the "blocking" antibody acquires an agglutinating function, however, when certain nonspecific protein materials are present in the diluent. There is, accordingly, evident variety among the antibodies produced in the human being. Development of quantitative methods of study that would allow resolution of mixtures of antibodies of like specificity may afford answers to some current problems.

Among the lower animals, analogous situations are found. "Low-grade" antibody has been recognized in immune sera prepared in the rabbit (Heidelberger and Kendall, 1935; Heidelberger, 1947), being nonprecipitating when isolated but, in the unfractionated immune serum, participating in antigen-antibody reactions along with the other antibodies. Similar low-grade antibody occurs in immune serum produced by the horse, and in some bleedings it has been found alone, unaccompanied by any frankly precipitating antibody (Pappenheimer, 1940).

Furthermore, it is of interest that the horse produces two varieties of anti-protein precipitating antibodies, occurring in electrophoretically different fractions of serum, one precipitating in ordinary fashion, the other precipitating with antigen only in a very limited range of proportions, like equine antitoxin (Treffers, Heidelberger and Freund, 1947).

Proof of the occurrence in animal sera of reagins as a separate and distinct type of antibody is unsatisfactory, but there is presumptive evidence, and certainly demonstrable variety among the antibodies. In the scattered cases of clinical allergy occurring in animals—horses, cattle, dogs—antibodies capable of sensitizing the skin of other members of their species are often found (Reddin, 1948) and therefore are termed "reagins," usually without any critical examination, or information on thermolability. And similar transfer effects in guinea pigs can be secured with sera obtained by artificial immunization (Dienes, 1928; Ramsdell, 1928; Chase), those examined having been found to contain a high content of anaphylactic antibody and to exhibit no marked thermolability (Chase, 1947); the relation of the skin sensitizing



antibody to precipitin content of the sera has not been studied adequately. All these reactions are of the evanescent type. If it is valid to recognize "reagin" by ability to sensitize the human skin, in the absence of information on thermolability, it is evident that certain samples of animal immune sera have this property, and it does not always seem to correlate with precipitating antibody (Caulfeild, Brown and Waters, 1937; Winkenwerder et al., 1939; Sherman et al., 1939; Brunner et al., 1944).

As mentioned before, the transfer of Arthus-type sensitivity in rabbits appears to be referable to antibodies that react freely *in vitro* with soluble protein antigens (precipitins), and a like situation may be anticipated to hold with other rabbit sera that give transfer effects, e.g., to pneumococcal protein with sera prepared by the intravenous injection of heat-killed pneumococci (Julianelle, 1930).

#### THE RELATION OF CHRONIC ANAPHYLAXIS TO DISEASE

In the hope of elucidating certain disease mechanisms, anaphylactically sensitive animals have been stimulated with antigen repeatedly and examined for evidence of chronic changes. It is necessary, of course, to allow for the temporary desensitization that occurs in the wake of each injection. Often large amounts of antigen have been used to bring forth maximal responses. In recent years, largely through the work of Rich, the changes encountered have been linked to periarteritis nodosa and perhaps to other diseases of man.

Repeated injections of antigen into sensitized dogs, cats, rabbits and guinea pigs were found by Longcope (1913) to affect, according to routes of injection and the species, kidneys, myocardium, liver, lungs, peritoneum and omentum. In extreme cases the alterations "produce the picture of an extensive myocarditis, a periportal cirrhosis of the liver, or a widespread subacute nephritis" affecting principally the epithelium of the tubules with

occasional involvement of the glomeruli; kidney damage was the most constant finding. These studies were confirmed by Boughton (1916, 1917, 1919), who pointed to the occurrence of subacute vascular damage of the small arterial vessels (endothelial degeneration and regeneration, and edema), principally in the liver and kidney of the guinea pig and in the spleen, lung and heart of rabbits. More severe changes owing to the injection of large amounts of antigen (15 to 20 cc. serum) into sensitized rabbits were studied by Vaubel (Klinge, 1933), by Apitz (1933) and by Bruun (1940), definite injury being seen at times in myocardial vessels, while Junghans (Klinge, 1933), working with pig serum—a material having high primary toxicity—encountered even granulomas of the coronary vessels, aortitis and endocarditis.

When normal animals are given a single injection of a large amount of foreign protein, this will not only induce sensitivity but by persistence of antigen (cf. Hawn and Janeway, 1947) may cause, within one to three weeks and at about the time of the appearance of antibodies, changes in the parenchymatous organs that are similar to but less intense than those occurring when small amounts are reinjected (Longcope, 1915). Another sort of reaction, an erythema and edema occurring in the rabbit's ears usually 5 to 8 days following a single large injection of foreign protein, frequently accompanied by hyperpyrexia, has been observed by Fleischer and Jones (1931) (cf. Kellett, 1930), and is to be interpreted, as they remark, as an analogue of serum sickness in man.

In an experimental study of arthritic changes, Klinge (1933) made injections of antigen, such as horse serum (0.25 to 1.0 cc.), into the knee joint of rabbits sensitized by the Arthus procedure, and found not only violent allergic inflammation in the adjacent soft tissue, with necrosis and collagenous degeneration, but also inflammatory-degenerative alterations in the heart muscle and valves, blood vessels, voluntary muscle, tendons, etc., a distribution which the author relates to sites affected in rheumatism. When these injections were made repeatedly into the same joint, a severe destructive and deforming arthritis occurred. In some cases, however, as when Seegal and Seegal (1931) kept the eye of a sensitized rabbit in a state of allergic inflammation during 8 days, repeated antigenic stimuli have not led to permanent tissue damage.

A further advance was made when Rich (1942, 1945, 1947) emphasized, after careful histologic examination, the likeness between changes in human beings and in rabbits in consequence of treatment with antigens. In the organs of patients experiencing recent hypersensitivity reactions due to foreign serum and sulfonamide, or to iodine medication, he found fresh vascular lesions characteristic of periarteritis nodosa, very probably a manifestation of the hypersensitivity. Rich and Gregory (1943) then were able to show arterial lesions typical of human periarteritis nodosa (cf. Hopps and Wissler, 1946), as well as the usual acute diffuse glomerulo-nephritis, in rabbits that had been given large initial intravenous doses of horse serum (10 cc./Kg.), then usually subsequently restimulated with antigen, and finally sacrificed between 1 and 7 days after the last injection. The hypersensitivity both in man and in the rabbit was considered to be of the anaphylactic type in that it affected chiefly blood vessels, although equivalent primary necrosis of the larger arteries is not met with in the rabbit; it is stated that the "arterial lesions, both in their focal distribution and in their origin as foci of localized edema, strongly suggest that periarteritis nodosa represents, in effect, 'hives' of the blood vessels . . ." By further careful study of the tissues of many rabbits that were treated in the same way with large doses of antigen, Rich and Gregory (Rich, 1947), following Klinge and others, found in some of the animals valvular and myocardial lesions, small focal lesions and thrombosis in the alveolar capillaries, narrowing of the lumen of affected arteries by intimal proliferation, and other changes, bearing variously a decided resemblance to rheumatic carditis, rheumatic pneumonitis, or to coronary "sclerosis" of the sort seen in rheumatic fever and in disseminated lupus erythematosus. As to whether Aschoff bodies, which occur in the connective tissue of human heart muscle and are characteristic of rheumatic fever, also occur in the rabbit, it is worth mentioning that scattered perivascular myocardial lesions are to be found in this animal and Rich considers them as being suggestively like Aschoff bodies.

Emphasis is placed on the anaphylactic-like character of these lesions, as seen both in the rabbit and in man: there would be one basic mechanism, according to Rich and some other workers, for periarteritis nodosa,

rheumatic fever and pneumonitis, disseminated lupus erythematosus, and rheumatoid arthritis. Whichever anaphylactic event would occur in a given human case would be referable primarily to individual predisposition and the peculiarly elective sites in which either the antigen becomes fixed (Hawn and Janeway, 1947) or antibody is produced. Despite striking analogies, including the facts concerning periarteritis nodosa, the thesis is not proven that these diseases or even some cases of them are referable to sensitization, for the variety of reactions which tissues exhibit is very limited (Klemperer, 1947; Bohrod, 1947) and the response of tissues to injuries of different origin may be quite similar. It has even been suggested (Cooke, 1947) that delayed allergic reactions as well may play a role in vascular diseases such as periarteritis nodosa.

Working in the manner of Rich but using isolated beef gamma globulin and albumin in addition to whole serum, Hawn and Janeway (1947) administered single large doses and found acute lesions only when antigen was still present and before antibody appeared in the circulation; this circumstance poses a new question for investigation. The two serum proteins gave rise to different localizations of lesions—albumin to arterial lesions, gamma globulin chiefly to kidney and nonarterial heart lesions. The times required for the lesions to develop varied according to the inciting protein, and were similar to the respective incubation periods observed before onset of the human serum sickness induced by these two materials—lesions due to albumin were maximal in 2 weeks, those due to globulin were at their height in only 1 week.

In short, the studies of Rich and of other recent workers would strongly suggest that anaphylactic reactivity in man may at times be prominent only in particular sites. A situation analogous to this has been sought in animals, namely the existence of localized areas of sensitivity, and there is evidence that such a type of "local anaphylaxis" may occur.



A local area of sensitiveness has been produced experimentally in the rabbit's eye by Von Szily and by Seegal and Seegal (1931), the eye sensitized by previous injection of antigen into the anterior chamber exhibiting hyperemia, edema and lacrimation upon a later intravenous injection of specific antigen and even sometimes (with a time-lag) upon introducing the antigen by stomach tube. Likewise, placing antigen in the pericardial sac of the normal rabbit proved to be more regularly effective than other routes in engendering a sensitization affecting the coronary vessels of the heart (Seegal and Wilcox).

In addition, under special circumstances localized inflammatory responses may occur in generally sensitive animals owing to some unusual distribution (and concentration) of antigen. For instance, unilateral glomerulonephritis in rabbits has appeared following the injection of bacterial cells directly into the renal artery, apparently because of local retention of bacillary antigen in high concentration (Lukens and Longcope, 1931). In the same category, apparently, is the elective destruction of normal kidney tissue following an injection of organ-specific antibody, e.g., injection into the rabbit of antibodies for rabbit kidney that were prepared in the duck (cf. Kay, 1940).

Local tissue damage or alteration, also, can be a factor in the production of lesions: heating, or chilling, or local irritation of the skin with xylol will all permit circulating antigen to produce local lesions in sensitized rabbits (Opie, 1936; Klinge, 1933). Injury is a factor in the Shwartzman phenomenon, too (page 148).

#### ALLERGIC INFLAMMATION: DELAYED RESPONSES

We will now consider those allergic reactions that require some hours to become manifest after the test material is deposited in or on the tissues. These reactions not only develop more slowly than do those of the "early" variety, but they exhibit no readily demonstrable relation to circulating antibodies, and the procedure of passive transfer by means of serum is typically unsuccessful. Nonetheless, the reactivity is specific, and a relation to an antibody mechanism of some sort is hardly to be

doubted. Analysis is further confused by the coexistence, in some cases, of both the early and the delayed types of response.

"Delayed" responses as seen in the skin may involve epidermal cells alone or the deeper tissue layers, according to the manner of sensitization, and consequently various dermatologic classifications have been erected (Sulzberger, 1940). Epidermal sensitivity is induced chiefly by contact of the skin with substances of low molecular weight (drugs, nickel salts, urushiol from the poison ivy plant and the like) or with products of fungi; upon a subsequent contact of the same, or a chemically related agent, epidermal reactions of eczematous type appear, with such lesions as macules, papules, or vesicles, and with hyperemia and itching; there is evident spongiosis in the tissues. A sensitiveness apparent in the deeper layers of the skin results from invasion of the body by a variety of infective agents (bacteria, fungi, parasites, viruses, etc.) or from the use of drugs. In the case of so-called "allergy of infection," usually termed simply "bacterial allergy," the sensitivity is detected by injecting various extractives or metabolites of the infectious agent (or entire viral suspensions) into the skin (cf. Jadassohn, 1932).

Recently, a basic relationship is becoming recognized between the quite different manifestations of certain dermatitides such as contact dermatitis and the tuberculin type of allergy, and it seems entirely probable that such various hypersensitivities share in a common cellular mechanism. We shall consider first the hypersensitive state attributable to infectious processes.

Evidence of hypersensitiveness of the delayed type was observed by Jenner in 1798 with the virus of cowpox: individuals inoculated with cowpox virus later responded to this or the closely related smallpox virus with only a small local redness that appeared within 48 to 72 hours and then faded quickly. This was interpreted as an allergy by von Pirquet and Schick in 1903:

the role of hypersensitivity was convincingly demonstrated when Hooker (1929) injected heat-inactivated virus into the skin of cowpox-immunized individuals. The outstanding example of reactions of delayed type was provided by Koch's discovery in 1891 of the tuberculin-tuberculosis relationship, but the underlying allergic basis was not recognized until 1903 (von Pirquet), and because of several confusing factors a clear distinction between the tuberculin reaction and reactions of the anaphylactic type seems to have been drawn only much later (Coca, 1920; Calmette, 1920; Zinsser, 1921).

#### TUBERCULIN HYPERSENSITIVITY

Koch's experiments with guinea pigs that had been rendered tuberculous showed plainly that infected animals possessed a special reactivity towards reinjection of the bacterium if the infection had been established for two weeks or more. Upon attempted superinfection by the subcutaneous route, there occurred an unexpected and massive inflammatory reaction of the tissues, which walled off the injection depot and usually led to slough—the "Koch phenomenon" (Chapter 12). Since this reaction did not require living cells, Koch sought a bacterial extract possessing the same property, and attained it by heating the mycobacteria for some hours in the medium in which they had grown (meanwhile concentrating the fluid), and finally removing the bacterial residue. This preparation was called O.T., or old tuberculin. When this product was injected subcutaneously, it was rapidly absorbed and caused a severe to lethal shock of delayed type, termed the "systemic reaction." Upon necropsy, *local* and *focal* reactions were found: locally along the needle track and in the subcutaneous depot there were hemorrhage and pronounced edema, the tissues appearing bluish red and rather gelatinous, and the draining lymph nodes enlarged and dis-

colored; there was hemorrhagic exudate in serous cavities; and everywhere the existing tuberculous lesions exhibited focal inflammatory reactions with enormously dilated capillaries and dense leukocytic infiltrations. The focal reactions seem to be responsible for the general toxemia and death.

The mechanism of tuberculin shock was not understood for some time, but in 1903 von Pirquet advanced the hypothesis that the reaction was another of the phenomena of sensitization, and this belief led him to the discovery in 1907 of the cutaneous tuberculin reaction, so important diagnostically (Chapter 12). Almost simultaneously, a delayed type of ocular reaction was noted as well. Tuberculin, originally hailed as a "Heilmittel" or curative and employed rather freely in its early clinical trials, caused many severe reactions and deaths because of its properties of exciting reactions in tuberculous lesions. Even in diagnostic testing by intracutaneous injection (Mantoux), it is necessary to select the dosage with great caution, for an excess of tuberculin may cause not only unduly severe reactions in the skin, but also, in some degree, "lighting up" around tuberculous lesions, and mildly febrile systemic reactions. The subject of allergic reactions to tuberculin is presented in detail by Tytler (1930).

When diluted tuberculin is injected into the skin of the tuberculous animal, there is no immediate and obvious reaction; but after a few hours, redness appears at the injected site, and the local inflammation with its associated edema gradually increases in intensity and somewhat in area for 15 to 48 hours, eventually with typical firm induration; meanwhile, if the dose and the degree of sensitivity allow of a severer reaction, there has been central blanching and the gradual development of an innermost livid zone, often becoming necrotic. The inflammation then slowly fades, but the lesion is palpable for some days and



pigmentation may be seen for several weeks. The reaction in man is much the same, but it shows a more prominent outer zone of diffuse erythema (Plate 1).

Histologic differentiation between the anaphylactic and the tuberculin types of response is possible in guinea-pig skin (Laporte, 1934) and in man, so far as it is a question of comparing evanescent and tuberculin reactions. In the anaphylactic type, the primary injury involves the blood vessels, and thrombosis and hemorrhages are frequent; centrally there is a relative paucity of wandering cells, and these are not principally mononuclear. In the tuberculin type, in contrast, the reaction develops slowly, the primary injury is in the epithelial layer of the skin, and there is a marked ingress of wandering cells, first polymorphonuclear cells (cf. Follis, 1940) and finally mononuclear cells which become the predominant type. But probably it would not be possible to find differentiating features in man between weak tuberculin reactions and weak reactions, apparently of Arthus type, that are often seen in known instances of sensitization to soluble proteins (McCarter et al., 1938).

In a careful study of the reactions of guinea-pig skin, Laporte found that, with the exception of progressive necrosis, the tuberculin type of reaction exhibited hardly any special character not to be recognized in principle, even if diminutively, in the inflammatory sequence initiated in normal animals by irritating substances and by tuberculin. In the tuberculin reaction, there occurs, histologically, early edema, capillary engorgement, and diapedesis of cells, chiefly polymorphonuclear leukocytes including eosinophiles. The cellular infiltration becomes broader and more intense, whereas the early edema and vascular congestion diminish; clusters of polymorphonuclear cells accumulate beneath the epidermis, being particularly dense in areas that will become necrotic. These are followed by monocytes, principally macrophages, which gradually become the predominating cell. The edema and congestion reappear. With strong reactions, signs of degeneration in the epidermal cells may become evident around the eighth hour; the necrosis progresses and includes, in more or less degree, the superficial layers of the skin and the deeper fat cells; it may extend deeply and appear hemorrhagic. Below the necrotic zone, fiber bundles

are swollen and turbid and there is a fibrinous edema. The reaction reaches its peak shortly after 24 hours. In the following days, just as happens after strong anaphylactic skin reactions or other forms of severe early irritation, histiocytic granulation tissue forms in the deep layers of the skin, and giant cells and lymphocytes are seen (cf. Stewart and Rhoads, 1926).

One of the early difficulties in interpreting the tuberculin reaction had to do with the occasional demonstration of an anaphylactic state in tuberculous guinea pigs. This could be shown, irregularly, by injecting intravenously a watery extract of tubercle bacilli in place of the long-heated product, old tuberculin (Baldwin, 1910). In other ways also, as by complement fixation tests with serum and *M. tuberculosis*, the presence of circulating antibodies was known. The concentration of such antibodies did not fluctuate with the intensity of the tuberculin reaction, and when the skin reactivity was made to disappear by administration of a considerable quantity of tuberculin (temporary desensitization), the amount of circulating antibody was only slightly reduced (Freund and Mansfield, cited by Opie, 1936). The situation is still confusing, but it is evident that two states, namely anaphylaxis and delayed type sensitivity, can occur separately or together.

In order to secure a truer picture of the tuberculin-tuberculosis relationship, attempts are being made to examine the constituents of the tubercle bacillus as they may be found in unheated culture filtrates (Seibert and Nelson, 1943; Bevilacqua and McCarter, 1947) or secured from the cells upon extraction or following autolysis; one heat-coagulable protein of large molecular size (ca. 44,000), hardly to be detected in old tuberculin, has been demonstrated. At the same time, it should be understood that for the purpose of human skin testing it is desirable to elect low-molecular and even heat-altered material, so long as it is serologically reactive, in order to avoid the de-





## PLATE 1

### Representative types of skin reactions.

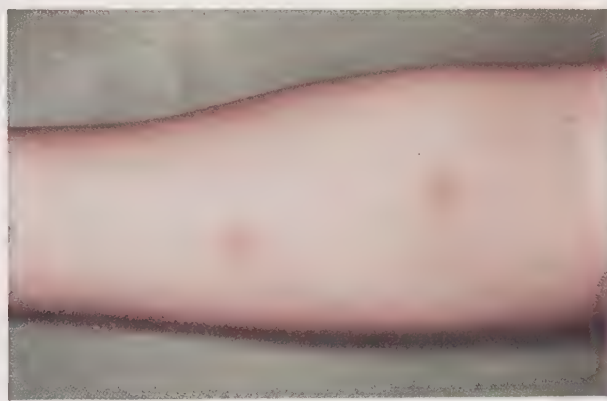
(*Top row*) Positive Schick reaction, as seen at 2 days (left) and at 4 days (right). Two test injections have been made, one of pure diphtheria toxin (right, near antecubital space), the other of toxoid prepared from the same toxin by formaldehyde treatment (left, toward wrist). Only the toxin gives a positive reaction. This is not an allergic reaction, but is due to primary toxicity of the test substance for skin, in which a *slowly developing* injury is produced. The detoxified material does not injure normal skin. (When neutralizing antibody is present, such injury to the injection of toxin does not occur, and the test is "Schick negative.")

(*Second row*) Pseudoreaction in Schick testing, as seen at 2 days (left) and at 4 days (right). The same two test injections have been made as above. Both the toxoid and the toxin have induced like reactions, differing in type from the simple Schick reaction shown above. These reactions are allergic in character and are due to the existence of a tuberculin type of sensitization to the protein molecule, independently of the integrity of the toxic grouping in the molecule. The reactions are maximal within one to three days, and then fade, leaving residual pigmentation. Compare with tuberculin test, bottom row, left. The subject is "Schick negative," as seen from the absence of progressive injury to diphtheria toxin and as known by measurement of antitoxin in the serum. Interpretation of the reaction to toxin could be confusing, however, if the *necessary toxoid control had been omitted*.

(*Third row, and bottom, left*) Positive tuberculin reaction, as seen after 11 hours (third row, left), after 27 hours (third row, right), and after 8 days (bottom row, left). Test injection with 0.2 microgram of tuberculin preparation PPD-67-2 (Seibert; cf. McCarter and Watson, 1942). The reaction became visible after some hours and was sharply defined by the eleventh hour; the site of a test made one week previously with 0.02 microgram is also to be seen. At 27 hours, the area was sore upon palpation and the reaction was accompanied by lymphatic "streamers" (lymphangitis) and tenderness in the regional nodes. The reaction was maximal at 35 hours, after which it regressed slowly. Evidence of the reaction was visible for some weeks (cf. bottom row, left).

(*Bottom, right*) Positive Prausnitz-Kuestner (flare-and-wheal) reaction. The serum of an individual subject to allergic coryza in encountering birch pollen was deposited (0.05 cc.) in the skin of a normal individual. One day later, an extract of birch pollen was injected into the center of the prepared area. The site is shown after 5 minutes. Note the central "wheal," blanched, elevated, and sharply circumscribed, surrounded by a broad erythematous "flare." The reaction had faded markedly 40 minutes later.

(Top four supplied by A. M. Pappenheimer, Jr., Photography Edgar J. Nebel.)







velopment of protein sensitivities through repeated skin testing.

Typical tuberculin sensitivity can be induced by the injection of killed cells, more readily by far when they are suspended in hydrocarbons such as paraffin oil (Zinsser and Petroff, 1924; Saenz, 1935; Flahiff, 1939). In such sensitizations, anaphylactic events are encountered more often than in infection. When separated tuberculoproteins are injected into animals, only the anaphylactic state becomes established.

Desensitization of tuberculin reactivity has been referred to above. It occurs, usually incompletely, in tuberculous men and animals after a single large injection of tuberculin has produced severe constitutional reactions. Desensitization is considerably more difficult in tuberculin sensitivity (and peculiarly hazardous because of the danger of focal reactions) than it proves to be in anaphylaxis. But, as Rich (1944) says, desensitization in man by repeated, gradually increasing doses of tuberculoprotein "has often been carried to a point at which the patients were able to tolerate, without a focal or constitutional reaction, doses of the protein which, in the undesensitized body, would undoubtedly have produced extreme focal reactions and death." The "desensitization" of tuberculous guinea pigs is a difficult and laborious task, requiring large, repeated doses of the tuberculin preparation "P.P.D." (Chapter 12), but during the treatments the skin is maintained truly nonreactive and it even will react to intradermal spread of dyes in the same way as does normal skin (Birkhaug and Berle, 1945); however, sensitivity returns when administration is discontinued. It may be added, as emphasized by Rich, that both in the occurrence of specific, temporary desensitization and in the accelerated rate at which tuberculin hypersensitivity has been observed to reappear upon a subsequent reinfection there is a marked

resemblance to the known behavior of the antibody-producing mechanism; these features have been cited as evidence that an antigen-antibody interaction underlies the effect of tuberculin on sensitive tissues.

Sensitivity to tuberculin is widespread among the cells of the tuberculous animal, and this sensitivity is evident in tissue culture, even in migrating individual cells and apparently in their descendent or "daughter" cells, at least of the immediately succeeding generations. (Rich and Lewis, 1928, 1932; Aronson, 1931; Moen and Swift, 1936; Moen, 1936c; Heilman, 1944.)

As Rich and Lewis observed in their classic paper (1932), when proper concentrations of tuberculin are used, "the damage done to allergic cells by tuberculoprotein is marked and easy to observe. It is evidenced by a decided inhibition of migration of the allergic cells from the explant into the surrounding tuberculin-containing plasma and by the fact that the relatively few cells which do wander out die in a few hours . . . Cellular injury and necrosis associated with allergy in tuberculosis result from a change in the individual fixed tissue and blood cells . . ." Both guinea-pig and rabbit tissues from experimental tuberculosis have been studied; the cells found sensitive are macrophages and fibroblastic growths developing from mononuclear exudative cells, and granulocytes. The injury to the migrating cells appears to be cumulative over a period of some hours. In contrast, an early effect on mature lymphocytes from tuberculous mice and guinea pigs has been observed by Favour (1947), a decrease of between one fifth and one half in number of intact lymphocytes being apparent in one hour or less after tuberculin is added.

In the intact animal, the relatively avascular cornea of tuberculous guinea pigs reacts to direct injection of tuberculin (Holley, 1935; Rich and Follis, 1940) with edema, swelling of the fibers, infiltration with granulocytes, and necrotization of corneal cells. It may be mentioned, as one differentiating feature, that no similar type of cellular reactivity has been obtained in the Arthus-sensitized rabbit (Rich and Follis, 1940) or in cultures of anaphylactically sensitized guinea-pig tissues (Aronson, 1933).



## ALLERGY IN OTHER BACTERIAL DISEASES

As mentioned, other bacterial infections show much the same sort of delayed-type reactions when extracts of the corresponding bacteria are put into the skin; such extracts may at times give immediate-type reactions as well, particularly in relation to a content of specific polysaccharides. Many of the delayed-type reactions are less pronounced than in the tuberculin-tuberculosis relationship, for as Boyd has remarked, "tuberculin allergy is one of the more extreme examples of bacterial allergy." Also, desensitization of the delayed type of reactivity is much easier to attain by repeated testing, and pronounced systemic reactivity may be uncommon.

Thus, when large samples of the population are tested with culture filtrates of group A streptococci (human pathogenic forms), there is found to develop with age a gradually increasing incidence of sensitivity and, superimposed upon this whenever diseases intimately associated with streptococci occur, a sharp rise in the number of reactors. This has been shown with culture filtrates of *Str. scarlatinae* by MacKenzie and Hanger (1927) and with nucleoprotein from hemolytic streptococci by Coburn (1931). In animal infections with group C streptococci, there are wholly analogous findings, e.g., skin reactivity in guinea pigs that are ill with epizootic lymphadenitis or are healthy carriers of the responsible micro-organism (Moen, 1936b), and reactivity in rabbits to extracts of their natural pathogen *Past. cuniculicida*, the organism causing "snuffles" (Hanger, 1927).

It is more pertinent, for our purposes, to examine hypersensitivities that have been induced deliberately by experimental methods. One of the earliest observations was that of Gay and Force (1914), who found a skin reactivity to "typhoidin," an extract of *S. typhosa*, after injection of human beings with typhoid vaccine or following recovery

from typhoid fever. In lower animals, allergy to streptococci has probably been best studied. Zinsser and Grinnell (1925) induced a skin sensitiveness to culture filtrates by injecting living streptococci into the peritoneal cavity of guinea pigs, and in the same year the first of a series of comprehensive studies on the sensitization of rabbits appeared from Swift's laboratory.

It was shown by Derick and Swift (1929) that rabbits could be sensitized with *viridans* streptococci by intracutaneous injection and by several other routes, with the notable exception of intravenous injection. In the latter case, a type of immunity became established, but otherwise, and apparently in connection with the development of some focal tissue reaction, the animals presented cutaneous, ophthalmic and systemic hypersensitivity to the administration of living streptococci, all being expressions of a delayed type of reaction, unrelated to the amount or type of circulating antibody and not transferable by serum; furthermore, the inception of such a type of sensitivity was often heralded by the appearance of a secondary reaction, or allergic "flare," at the site of primary injection after 7 to 9 days. When rabbits thus sensitized were tested with the separated nucleoprotein or polysaccharide fractions of the cocci (McEwen and Swift, 1935), tuberculin-type responses were secured but in addition the reaction to the nucleoprotein started as an immediate response and had an edematous as well as an indurated character. This appears to suggest a coexistence of both sorts of skin effects. In contrast, rabbits sensitized by means of the nucleoprotein responded only with an immediate type, anaphylactic reaction.

To relate further the mechanisms of streptococcal and tuberculin hypersensitivity, Moen (1936a) examined both sorts of sensitivity in parallel, in tissue culture, using explants of tuberculous tissues and explants of the spleen of guinea pigs infected with a natural streptococcal patho-

gen (group C hemolytic streptococci from guinea pig lymphadenitis). The presence of streptococcal extract led to a slowly developing specific toxic effect on the cells of streptococcus-infected animals, quite similar to but quantitatively less than that of tuberculin on tuberculin sensitive cultures. The streptococcus-sensitive cells exhibited, rather more pronouncedly, various gradations of sensitivity, some cells being killed rapidly, others being only slightly inhibited. Very much less evidence of persisting sensitivity was seen in subcultures of the streptococcus-sensitive cells as compared with explants of tissues from tuberculous animals.

In pneumococcus pneumonia, two types of specific skin reactions have been observed (Tillett and Francis, 1929): one due to the type specific capsular polysaccharide, appearing at the onset of convalescence and consisting of an immediate reaction of urticarial type, the other due to species specific nucleoprotein and being of the tuberculin type (see p. 104).

In addition to these two reactions, a third variety, described more fully in Chapter 5, has been disclosed (Abernethy and Francis, 1937). The unique situation here presented, a skin reaction not based upon an antibody mechanism, is well worth noting. Not only in pneumonia but in a variety of other acute infections there appears temporarily in the blood a special protein, which is sharply differentiable by serologic means from any of the normal blood proteins (Abernethy and Avery, 1941; MacLeod and Avery, 1941). This peculiar material, which has been isolated and crystallized, happens to react in vitro with the somatic "C" polysaccharide of pneumococci, and the patients in whom it is found give chiefly a delayed type of skin reaction when they are tested with this polysaccharide; their serum loses the capacity to react in vitro upon heating above 65° C. It may indeed be supposed that, except for keen experimental study, the reaction would be attributed to an antigen-antibody interaction.

Pneumococcal allergy has been studied in rabbits in much the same way as has

streptococcal hypersensitivity. In rabbits immunized by deposition of vaccine in the skin, one finds a delayed type of skin reactivity toward pneumococcal vaccine or nucleoprotein and a delayed type of ocular reactivity (Julianelle, 1930; Harley, 1935, 1937); by means of serum the manifestation of reactivity to nucleoprotein is passively transferable and apparently is of Arthus type, while neither the allergy to vaccine nor the eye reaction is so transferable.

With both nonhemolytic streptococci and encapsulated pneumococci, intravenous injection of intact cocci leads to the development of anticarbohydrate antibodies and to immediate-type reactivity expressed toward the corresponding specific polysaccharide.

So far as there may be a relation between bacterial allergy and the production of disease, it may be stated that experimental studies on bacterial allergy have been interpreted as suggesting some parallelism between the hypersensitive state to streptococci and rheumatism, and between the "immune" state and bacterial endocarditis. Forms of arthritis and endocarditis have been made to appear in horses by first immunizing them highly and then introducing the bacteria into the blood stream (Bieling, 1930, 1931).

The idea that an individual may be sensitive to the products of bacterial strains harbored within some focus in his own body (antrum, root canal or socket of tooth, Bartholin gland, throat, etc.) has been nurtured for long. Evidently the process may be at play in streptococcal pyoderma of children. Cooke (1947) has cited scattered cases of infective asthma and of recurring intrinsic dermatitis in which the injection of autogenous vaccine precipitated asthmatic crises or exacerbations, and in which the removal of bacterial depots by physical means or by use of antibacterial agents has brought permanent relief. Diagnostic skin tests are usually of little value.

In the natural infection of man and horses with glanders and in experimental infection of the guinea pig, a delayed type of ocular reaction, skin sensitivity, and a systemic, general reaction (temperature rise) are expressed to mallein, analogous to the tuberculosis-tuberculin relationship. Likewise, in the infec-



tions of cattle caused by *Myco. pseudotuberculosis* (Johne's bacillus), there are skin reactions, conjunctival reactions, and generalized systemic reactions to "johnin," a preparation made like tuberculin and variously purified; a reaction occurs also with avian tuberculin. Johnin may be standardized by intradermal tests on hamsters and guinea pigs sensitized (but evidently not infected) by means of living bacilli in conjunction with substances which have an "adjuvant" effect, for instance paraffin oil or calcium phosphate (McIntosh and Konst, 1943).

A few other examples may be cited. Skin reactions have been employed to detect present or past infection in brucellosis ("brucellin," a culture filtrate, or "brucellergin," a nucleoprotein extract) (Burnet, 1922; Huddleson, 1939), in tularemia with chemically treated vaccines (Foshay, 1932), and in leprosy with lepromin, an extract of human leprous tissue.

In special cases, a similar sensitivity may be present with respect to antigenic bacterial toxins. As is well known, diphtheria exotoxin exerts a primary toxic effect on normal skin (the Schick reaction), this injury being averted, within limits, when neutralizing antibody (antitoxin) develops. But there may be evident in about one-fifth of young adults, not frequently in children, a tuberculin-type of sensitivity expressed against the toxin molecule itself [employed either as purified toxin or as formolized toxoid derived from pure toxin (Pappenheimer, 1948)] or against other constituents present in the less pure commercial preparations of toxin and toxoid. This tuberculin type of response, representing the so-called "false" or "pseudopositive" Schick reaction, lasts a day or so and then fades; it can commonly be differentiated from the true Schick reaction which appears slowly and develops increasingly during the course of four or five days. These various reactions are illustrated in Plate 1 and are discussed further in Chapter 9.

It is not our purpose to enter into a discussion of the diagnostic usefulness of each test of this sort, but rather to point out the underlying principle. To make any test practicable for diagnosis, accurate and statistically valid information must be had on such matters as the purification and proper concentration of the test agent, the most suitable manner of application, the degree

of correlation between preceding invasion and definite skin reactivity, and the relative specificity of the reaction. It must also be known whether the material employed for skin testing can lead to active sensitization and vitiate future tests. These points must be evaluated separately for each type of disease agent; even in the most studied case, tuberculosis, investigation does not cease. It seems true with many extracts that the ease of injecting skin has fostered the making of tests without adequate biochemical information on the material in the syringe. And particularly in those instances in which both early and delayed reactions can be shown it is important to evaluate each reaction independently.

In general, when the method of testing is specific, such tests have a significance similar to that of tuberculin; a positive reaction denotes the occurrence of infection but gives no reliable indication of current activity. It is most informative when the skin reactivity is known to have been acquired recently. One must recall, furthermore, that in advanced stages of all diseases, including tuberculosis, the skin may fail to react: this condition is called *anergy* and has been considered a consequence of an "exhaustion" on the part of the tissue cells. Also in intercurrent infection such as measles and chickenpox, the tuberculous host may temporarily cease to give skin reactions to tuberculin. Even a positive test which is specific may not always be of practical diagnostic value: for example, Sulzberger (1940) points out that interpretation of reactions to extract of monilia ("oidiomycin") must be weighed against the well-nigh universal exposure to various species of monilia.

#### ALLERGY IN NONBACTERIAL DISEASES

Following invasion with a wide variety of agents other than bacteria, an altered state of the body may be shown through an altered reactivity of the skin.

In fungal infections, the usual skin reaction is of the delayed type (24 to 48 hour reaction). Highly useful diagnostic results have been obtained in coccidioidomycosis with *coccidioidin*, an extract of ground, heated culture growth, and evidence of general systemic reactions have been seen. In histoplasmosis of man and of experimental animals, reactions are obtained with *histoplasmin* (culture medium which has supported the growth of *H. capsulatum* for several months). In trichophytosis, various preparations of *trichophytin*, an extract of triturated cultures with their metabolites, have been used; the common diagnostic reaction is of the delayed variety, but a few subjects have shown also immediate urticarial reactions and corresponding reagins, and in them skin testing has been known to bring on asthmatic attacks.

Delayed-type reactions have been secured with viral materials. The skin reaction shown in smallpox-immune persons by killed vaccinal virus (Hooker) has already been mentioned. The Frei test has been used diagnostically in cases of Lymphogranuloma venereum (injection originally made with diluted and heat-sterilized pus, now sometimes performed with infected mouse brain or antigen obtained from chick embryo cultures). And a skin test betokening infection with mumps virus has been developed by Enders et al. (1945, 1946), who used preparations of the parotid glands of monkeys infected with monkey-adapted virus.

In contrast to the above observations, the form of allergic response that has received chief consideration in parasitic helminth infestations—for example, schistosomiasis, echinococcus disease, filariasis, trichiniasis, and ascaris infestation—has been the immediate reactions of the wheal-and-erythema type found when the skin of the host is tested with extracts of the body substance of the same or related parasites; antibodies can usually be demonstrated in the serum by serologic methods at the same time. Asthma and rhinitis are sometimes elicited upon exposure to the specific agents. While there is often an early reaction to hydatid fluid in echinococcus infestation, it appears that the delayed type, or *Casoni reaction*, should be given chief diagnostic interpretation.

In protozoan infestations, skin tests have been less useful. The only instance to be mentioned is leishmaniasis, in which a tuberculinlike reaction has been observed in skin

tests made with an extract of cultured leishmania.

Even as an aftermath of sensitization by insect bites, delayed allergic reactions have been demonstrated (Benson, 1936) independently of early reactions (and corresponding reagins) which occur at times: both varieties may be seen in the same individual.

#### THEORETICAL CONSIDERATIONS

It is not known why bacterial infection leads characteristically, but not exclusively, to a "delayed type" of reactivity, whereas various extracts of the same bacterial cells, injected separately, give rise only to the anaphylactic type of sensitivity. It is all too common a practice to offer the explanation that bacterial cells contain multiple antigens which have different "antigenic characteristics," some giving rise to the anaphylactic state and others to the delayed type of reactivity. In one sense this conclusion is valid, for the former is often related to bacterial polysaccharides, the latter to bacterial proteins, but we must recall that after bacterial invasion, and more regularly after multiple injections of bacteria, an anaphylactic type of hypersensitivity to bacillary protein can at times be demonstrated (bacterial anaphylaxis versus protein), and it is probable that this occurs always even if inappreciably, along with the more obvious development of the delayed type of reactivity. Rather than regarding bacillary protein and nucleoprotein as having chemical and immunologic peculiarity, it seems more reasonable to consider that the invading micro-organism carries its antigens into a locus which becomes a special environment as host cells mobilize, and that the manner in which these cells deal with the slowly liberated protein antigens, being in some way different from the usual antibody-producing sites, determines the predominating, delayed type of sensitivity. There seems to be little cause to refer the occurrence of delayed type reactions to especially constituted antigens within the bacterial cell, for



indeed under special circumstances other, ordinary proteins can be made to give rise to the same delayed type of sensitivity.

For example, by the usual methods of injection egg white (as well as each of the proteins thereof separately) produces anaphylactic sensitivity, and corresponding transfer antibodies, in the guinea pig, but if it is injected into a tuberculous focus (Dienes, 1929) it gives rise to the characteristic delayed type of sensitivity as well as to anaphylactic sensitivity. In other words, the tuberculous animal comes to possess two coexisting sensitivities of the delayed type, one expressed when common protein (egg white) is injected, the other when a form of bacillary protein (tuberculin) is employed; both reactions are specific and independent, but are of the same reaction type. As Burnet (1941) has said, the induced, "tuberculin-type" hypersensitivity to egg protein "therefore seems to be definitely related to the placing of the antigen in an inflammatory area in which histiocytes and lymphocytes predominate." Similarly, when horse serum was included in a depot of killed, dried tubercle bacilli suspended in paraffin oil, Freund and McDermott (1942) obtained the Dienes effect to this protein, namely, a delayed type of reaction to horse serum; by the same device, Landsteiner and Chase produced a delayed type of reaction (contact dermatitis) towards such allergenic substances as picryl chloride by injecting, along with dead tubercle bacilli, insoluble conjugates of the sensitizing materials. It is therefore unlikely that the second antigen present in the lesion becomes modified chemically; rather, it would seem that the event which occurs in the focus with regard to tuberculoprotein, and leads to a delayed type of sensitivity expressed towards tuberculoprotein, extends as well to other kinds of protein put into the lesion.

The intracutaneous route of injection is outstandingly successful in leading to sensitivities of the delayed type, for example with vaccines or living suspensions of bacteria or with allergenic drugs. There is reason to believe that the cellular response following intracutaneous injection may be such as to constitute focal reactions; in addition, the ready entrance of a part of the injected material into the lymphatics, and thence into the draining lymph nodes, may be of importance.

What determining event can occur in the focus is unknown. As a hypothesis, Burnet,

amplifying on the view of Rich and Lewis (1932), suggests that eventually there is produced and put into the blood stream a special antibody, of such variety that it binds to tissues with extreme readiness, and therefore will seldom be encountered in the blood in amount sufficient to permit passive transfer.

In any event, the basic importance of focal tissue alterations in determining immunologic response has been stressed by many workers, including those studying delayed reactions in diseases other than tuberculosis. As Swift and Derick (1929) stated, "In practically all instances in which a tuberculin-like allergy is induced this follows some focal tissue reaction resulting from injury by the respective microorganisms."

Recent observations point to a new experimental approach in the investigation of delayed-type reactions, and suggest that the underlying principles may finally become known. It appears not improbable, in line with the hypotheses reviewed above, that an antibody of a special sort is concerned.

In consequence of studies with guinea pigs made hypersensitive to certain chemical substances and the finding that the delayed type of reaction to chemicals can be transferred to a fresh animal by using washed cells of the donor instead of its serum (page 147), a similar technic was applied to the problem of tuberculin sensitivity. Guinea pigs rendered hypersensitive to tuberculin by prior injection of dead *Myco. tuberculosis* in paraffin oil were used as donors in the first experiments. Again it was found that living white cells gotten from peritoneal exudates, spleen, lymph nodes or blood would, upon injection into a new guinea pig, cause the latter to acquire a hypersensitivity to tuberculin for a limited period of time. The reactions developing in the recipient animals exhibited the essential features of the typical tuberculin reaction—erythema, central blanching, increasing induration, and sometimes superficial necrosis (Chase, 1945; Cummings et al., 1947; Kirchheimer and Weiser, 1947). When the experiment was repeated, using as cell donors animals infected with virulent human tubercle bacilli, the same result, both quantitatively and qualitatively, could be demonstrated, but inconstantly, probably for technical reasons related to the infectious process (Chase); Kirchheimer and Weiser (1947) employed

successfully guinea pigs that had been sensitized with living cultures of the slightly invasive strain, "B.C.G." An interpretation awaits further work. It appears unlikely that it is only the transferred cells that respond to later tests with tuberculin, for the dermis is everywhere sensitive, and an active participation of host cells in the response to tuberculin testing seems more likely. The result is strongly suggestive of an elaboration by the transferred cells of an antibody-like material. The literature on prior attempts to effectuate transfer is presented by Kirchheimer and Weiser; the experiments were not convincing because of wanting reproducibility, but it is probable that some actual transfers were attained. In many of these reports, tissue was included in the material transferred.

#### DRUG ALLERGY; CONTACT DERMATITIS

The manifestations of allergy to drugs are manifold, and can duplicate all the aspects of allergic reactions that we have been considering—anaphylaxis, immediate-type reactions, and delayed reactions. Among the facets of drug allergy are included such various forms as serum sickness, arthralgia, scarlatiniform and morbilliform rashes, asthma, flare-and-wheal reactions, urticaria, exfoliative dermatitis and many others. It is rare to encounter many forms in one individual, and, apart from inherited predisposing factors in the subject himself, the chief determining elements appear to be the frequency and mode of contact through which sensitization has arisen and especially (though seldom obviously) the chemical properties of the sensitizing material. More commonly encountered by far is the delayed type of reaction. The reader is referred to Sulzberger (1940) and to Landsteiner (1945) for reviews which deal with historical and theoretical aspects.

We will be concerned in this discussion not only with "drugs" (medicaments) given per os, or by injection, or by topical application, but with various materials of simple constitution ranging from the metal nickel to the catechol derivative of the poison ivy

plant, urushiol, which causes the allergy known as poison ivy dermatitis. It is to be understood that we are dealing with allergic manifestations, and not with exaggerated sensitivity to the established pharmacologic properties of the respective materials.

The causative agents are, plainly enough, not antigens, but it appears that by combining with the tissues of the host (either directly or after some intermediary chemical alteration *in vivo*) they acquire antigenic capacity and function somewhat like the "conjugated antigens" of Landsteiner, which arise when hapten structures become attached to indifferent foreign protein.

According to this concept, the newly formed complexes would effect the sensitization, and reactions would be elicited later whenever the same or a chemically related substance presented itself by the proper route.

This working hypothesis, derived from chemical and immunologic theory, would allow of several corollaries. As will be seen, these are not without experimental support.

The sensitizing contact, should it give rise merely to antigenic complexes that are soluble and are readily absorbed, would probably induce only an anaphylactic-type sensitization, just as we find when solutions of bacterial nucleoprotein are injected into animals. It is more to be expected, in order for the delayed type of reactivity to develop, that the formation of the sensitizing complex will occur in relation to some focal tissue reaction.

And, further, we could well expect that often both the delayed type of reaction and the formation of anaphylactic antibody would be induced simultaneously, even if wholly disproportionate.

Upon the eliciting contact, it is likely that mere contact between sensitive skin and the small molecule itself—analogue to the *in vitro* procedure of mixing antibody and hapten—would not suffice to induce a reaction, owing to the small molecular size of most of these allergens, but that a new combination of the allergen with proteins or other substances of the host would be a prerequisite. And from this point of view, the opportunity for observing "cross reactions" in making tests with a series of related chemicals would



be limited to those compounds whose chemical properties permit their joining with constituents of the body.

Experimental investigation may be said to date from 1896, when Jadassohn first published his careful observations and introduced the patch test as a diagnostic procedure in the study of contact dermatitis. For many years such allergies were considered to represent individual and innate idiosyncrasies: only a few people, for example, become sensitized to the primrose plant (*Primula obconica*). This idea was shown to be untenable when Bloch and Steiner-Wourlish (1926) pointed out that any individual could be rendered sensitive if one simply used a concentrated extract of *Primula* leaves and a sufficient number of applications. Some persons became sensitive after one application, others required several treatments, and evidently the role played by heredity was reflected in this. The same workers next showed (1930) that guinea pigs, as well, could be sensitized to *Primula*, and Mayer (1931) demonstrated that, like human beings, guinea pigs could be made sensitive to paraphenylenediamine, a compound often used in the dyeing of furs. The way was now open for animal experimentation, for the manifestations of dermatitis in man and in sensitized guinea pigs were analogous even if not identical.

At the cost of historical balance, we may simply summarize the chief results of experimentation (Landsteiner et al., Sulzberger, Haxthausen, Grolnick, etc.). Some of the chemicals that sensitize the human being will induce sensitivity in the guinea pig (2, 4-dinitrochlorobenzene, picryl chloride, picric acid, nickel salts, salvarsan, substituted benzoyl and benzyl chlorides, acid anhydrides, urushiol, and so on). In both species, sensitization is accomplished by repeated applications made on or into the skin, and it may be necessary to provide a previously irritated cutaneous area for the treatments. The sensitivity arises in from 5 to 20 days and is often seen first on the

seventh or eighth day as a "flare" in old sites, owing to a reaction with remaining traces of the sensitizing complex. The full degree of sensitivity is best demonstrated by applying the same material (usually dissolved in a suitable vehicle) to a fresh area of the skin, or by injecting intracutaneously either the excitant or an artificial "conjugate antigen" made by joining the incitant to some soluble protein.

These technics sensitize the skin generally, though in man there is some evidence for a slight delay before areas remote from the site of application acquire their sensitivity. There has been no experimental duplication of the so-called "fixed eruptions," in which sensitivity remains confined to certain fixed areas of the skin, differing from one patient to another (Sulzberger, 1940). In these cases it would seem that the skin reactivity is self-contained, for upon transplantation to a new locale the skin area is said to retain its capacity to react. The opposite situation, however, has been shown by Haxthausen (1943) in cases of general epidermal sensitivity that have been induced experimentally. Here it was found that upon skin transplantation (to a non-sensitive identical twin) the skin loses its sensitivity, while in the reverse operation nonsensitive skin acquires reactivity when grafted onto a sensitive twin. The loss of reactivity would seem to show that the skin need not be an important repository of antibody; the acquirement of reactivity would, entirely in keeping with other results to be mentioned later, merely indicate that there is an extra-epidermal mechanism for effecting sensitization.

Other findings with probable bearing on human allergy have come from the study of guinea pigs sensitized with simple chemical substances (Landsteiner, Jacobs, diSomma, Chase), and these suggest parallel studies with the human being.

The identification of sensitizing chemicals as those which can combine with proteins was demonstrated in the case of chloro- and

nitro-substituted benzenes (Landsteiner and Jacobs, 1936), and various classes of chemicals capable of sensitizing both guinea pigs and man have been found (Jacobs; di-Somma; cf. Gell, Harington, and Rivers, 1946); with most sensitizing substances, however, the basis of the combination with proteins remains obscure and may well reflect preliminary alteration *in vivo*.

It has been shown that through the sensitizing procedure, two different sensitivities arise in parallel—the anaphylactic type and the contact dermatitis type. The balance between these is largely controlled by the chemistry of the sensitizing compound: some compounds will produce chiefly a delayed-type contact dermatitis, others lead to both types, and still others give rise largely or exclusively to the anaphylactic type.

When sensitization is sufficiently intense, and there is available a proper protein conjugate of the incitant, systemic anaphylactic shock may usually be shown by injection of the protein conjugate into the blood stream, and, correspondingly, the serum will give passive anaphylactic transfer. Even more, the serum, simulating the behavior of “reagins,” may show the Prausnitz-Kuestner effect, that is, it can sensitize guinea-pig skin locally so that immediate-type reactions can be induced therein (Chase, 1947). This antibody may circulate undetected unless suitable protein conjugates are employed.

In contrast, the delayed type of dermal sensitivity, not transferable with serum, can be imposed upon normal guinea pigs by means of transferring washed, living white cells taken from sensitized animals (Landsteiner and Chase, 1942; Haxthausen, 1947); the cells, not improbably lymphocytes, appear to confer solely the delayed type of sensitivity. The induced sensitivity is usually demonstrable for a few days only. Such a mechanism would account for the acquisition of sensitivity by a normal guinea

pig when joined in parabiotic union with a sensitized guinea pig (Haxthausen, 1943).

This illustrates once again that immediate-type reactions and delayed-type reactions have different operational mechanisms, although an assumption of variant antibodies would be adequate to explain the phenomena. It is to be expected that the mechanism of transfer of the delayed-type reaction will depend upon immunologic principles now unknown and may be associated with intermediary processes in antibody formation.

In man, immediate-type, flare-and-wheal reactions to drugs have been found in several instances (salvarsan, formaldehyde, phthalic anhydride, chloramine-T, sulfathiazole, and sulfadiazine) and are often accompanied by demonstrable reagins; it is evident that these incitants, which are capable of giving early-type reactions, would largely belong in the category of the more reactive compounds. It may well be that, as in the case of sensitized guinea pigs, these reagins may occur more commonly than has been recognized, and that their detection may await upon the use of suitable protein conjugates.

Various attempts, not without reported success, have been made to secure a special variety of “cellular” antibody for purposes of passive transfer of the delayed type of reaction. The chief procedure employed (Urbach-Koenigstein technic) is to raise a blister on an area of skin that has recently undergone a specific reaction and to inject the blister fluid into the skin of a normal individual; the recipient is subsequently tested on the prepared site. This test is described by Urbach and Gottlieb (1946) and it is reported to yield positive results even in experimental sensitization with dinitrochlorobenzene (Ballesteros and Mom, 1945). The method does not appear to yield positive results with any regularity, but if it could be accepted, one would have a powerful tool to study the postulated new form of antibody.



Finally, it may be said that attempts at desensitization in contact dermatitis and in drug allergy have met with limited success; some cases recede spontaneously, a few others have been benefited by subcutaneous injection of the incitant in an oil vehicle (Caulfeild, 1936) or by cautious ingestion (Park, 1944; Stevens, 1945). A useful method has not yet been evolved, and except for spontaneous recession the sensitivity soon returns, as is true in all other types of allergic manifestation.

### THE SHWARTZMAN PHENOMENON

There remains to be mentioned a special sort of hemorrhagic, necrotic reaction that can be produced in the skin and some other organs (chiefly the kidney) but is not dependent upon an antigen-antibody mechanism: this is the phenomenon of local tissue reactivity developed by Schwartzman\* and described also under the name of the Sanarelli-Schwartzman phenomenon. In the original experiment, certain bacteria or culture filtrates of these were injected into the skin of rabbits and after a lapse of from 8 to 32 hours the same filtrate was introduced in quantity into the blood stream: within a few hours gross hemorrhage and necrosis developed at the prepared sites. It was found, however, that the "skin preparatory factors" and the "reacting factors" need not be identical or even immunologically related. For example, the local, preparatory injection may be made with culture filtrates of *E. coli* or *S. typhosa* and the intravenous, eliciting injection with meningococcal culture filtrates. Only certain bacterial strains will serve to provide adequate yields of "Schwartzman toxin" for the local preparation or "sensitization" of the skin; some strains are said to afford only the preparatory factor, others chiefly the reacting factor. For the local, non-specific sensitization of the skin, simple in-

flammatory reactions induced by a variety of irritants do not replace the use of selected bacterial filtrates. The active material in the culture filtrate is associated with bacterial protein—reputedly with the endotoxins or "complete" somatic antigens—and is antigenic. The rabbit is the animal of choice for this experiment; goats and horses show the effect, guinea pigs respond irregularly and less intensely, and mice and rats are not susceptible to skin preparation.

While both of the injections were originally made with culture filtrates of selected bacteria, either of the two may be replaced in various ways. It is possible to prepare the skin site with a bacterial culture filtrate and to elicit the reaction by an intravenous injection of starch (Freund and Hosmer, 1935). Or, if a skin site is prepared with culture filtrate in an immunized rabbit, the intravenous injection of the corresponding antigen will result, by means of an interaction between antigen and antibody functioning as the eliciting factor, in a typical necrotic lesion of Schwartzman type; this is not surprising, apart from the special qualities of the lesion that are a characteristic of the preparatory toxin and the delicacy of the reaction, since chilling or irritation of the skin under similar operating conditions can cause local allergic inflammation (page 136). The reverse situation, namely substituting for the preparatory injection a fully developed tuberculin reaction on a tuberculous guinea pig, and giving later an intravenous injection of a potent Schwartzman filtrate, also results in a typical Schwartzman reaction (Freund, 1934); tumor tissue in mice offers a situs for Schwartzman effects and responds with inflammation when potent bacterial filtrates are injected intravenously.

There has been active speculation that natural infections with bacteria may cause "tissue preparation" and that Schwartzman effects can occur due to reacting factors provided by the infection itself or by a variety of nonspecific stimuli; this has been surmised as being one possible factor operating in bacterial allergy, in the genesis of conditions such as pulmonary abscess, and the like. Proof, however, is still wanting for this view.

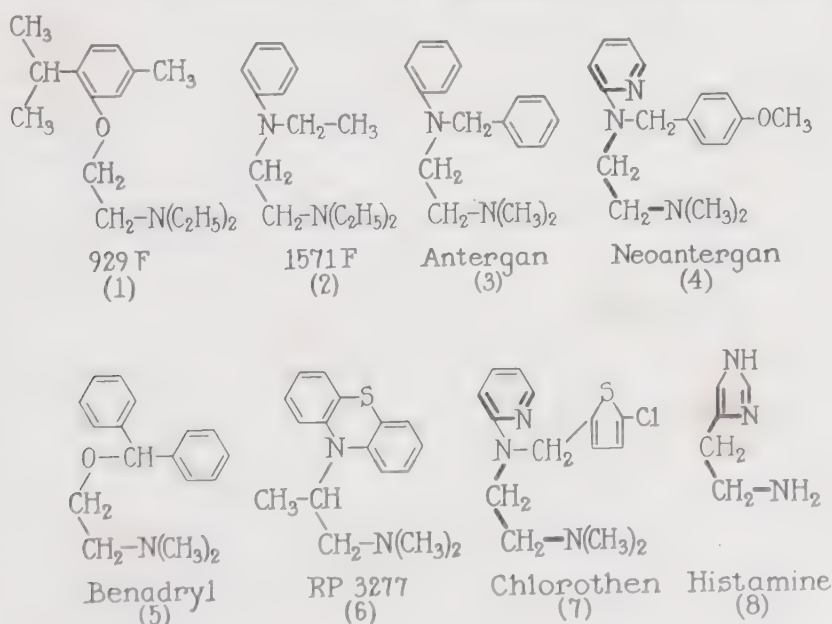
\* The reader is referred to Schwartzman (1937) for details of the reaction.

# "ANTI-HISTAMINIC" SUBSTANCES IN ALLERGY

The development of the histamine theory, emphasizing the role of histamine in systemic anaphylactic shock (pages 124, 125) and presumably in the allergic reactions of immediate type as well (page 129), has excited the hope that the physiologic consequences of antigen-antibody interaction might be avoided if the liberated histamine could be rendered ineffective in exciting tissue responses. Various approaches to this goal have been explored, such as administration of the enzyme histaminase, immunization by an "artificial antigen" containing histamine coupled to a protein, and, since 1937, administration of special compounds known to have "antihistaminic" properties. In extensive clinical trials made with the view of modifying or controlling allergic reactions, encouraging success has attended administration of the "antihistamines," although asthma especially is often resistant (Feinberg, 1946; Southwell, 1948). They are often effective in cases of allergic urticaria and allergic rhinitis, and they diminish the itching and certain other manifestations which accompany urticaria, angioneurotic edema, dermatographia, and serum disease; they may relieve the itching even in

poison ivy dermatitis. It is clear, however, that they "do not correct . . . all of the manifestations of histamine or allergic action nor do they relieve any particular symptom completely" (Feinberg, 1947), which is not unexpected if one takes the complexity of the factors in systemic anaphylaxis (pages 123, 124) as archetype of antigen-antibody reactions that occur in close relationship with tissues. Accordingly, the "antihistamines" serve as an adjunct and not as a panacea in the treatment of allergic conditions. There is little evidence that such "antihistamines" can modify in fundamental manner reactions that are of the delayed type. Although the compounds function also as local anesthetics, somewhat more so in this respect than procaine, there is evidence for dissociating this property from the antihistaminic effect. All these compounds can produce undesirable "side-reactions," as drowsiness, dizziness, disorientation, and gastro-intestinal disturbances; the effort directed to the development of compounds of intrinsically high antihistamine activity has been made with the idea of increasing the efficacy and reducing the side-reactions.

The stimulus to the synthesis of compounds having a protective action against histamine intoxication stems from 1937, when Bovet and





Staub detected such a property among certain compounds that had been prepared for other physiological studies; best of these was compound 929 of the Fournier series (see p. 149). Successive variation in its structure (see reviews by Feinberg, 1946, 1947) led finally to the announcement by Halpern in 1942 of *Antergan*, which possessed an activity sufficiently marked to be clinically useful. Following this, improved variants have appeared; it will be possible to mention only certain broad lines of development. *Neoantergan*, *Pyribenzamine* and *Chlorothen* among others are, like *Antergan*, derivatives of ethylene-diamine; other basic structures have been synthesized as well, including *Benadryl*, a benzhydryl alkamine ether, *Compound 3277 R.P.* containing a phenothiazine ring, and *Antistine* containing an imidazoline ring.

The evidence for the protective action of these compounds against the effects produced in a normal animal by administration of histamine is impressive; it extends to protection of the isolated guinea pig gut or uterine horn from contraction by histamine, and to inhibition of the vascular effects of histamine in the rabbit and other animals; to the protection of guinea pigs from multiple lethal doses of histamine, and from the bronchospasm caused by histamine aerosol. At the same time, and still without explanation, it is found that these compounds do not modify the gastric secretagogue action of histamine nor do they prevent the induction of gastric ulcer by histamine.

When the antihistaminic compounds are tested for ability to protect anaphylactically sensitized guinea pigs against systemic shock by an intravenous injection of antigen, larger amounts are necessary (3 mg./kg. down to 1 mg./kg.) than are needed to deviate the effects of purely histamine shock.

The structural formulas of some of these compounds are shown below, in comparison with histamine itself.\*

The "antihistaminics" do not neutralize histamine in vitro by combination, and apparently are effective only so long as they can be maintained in histamine-sensitive tissues at an

adequate level with respect to the molecular concentration of histamine. It is presumed that the principal pharmacologic activity of such substances lies in successful competition with histamine for histamine-sensitive tissues, owing to sufficient structural likenesses to permit a type of "competitive inhibition" (page 46). As support for this theory, Gaddum\* points out that the portion of the histamine molecule responsible for its physiological activity can be represented by the side-chain and a portion of the imidazole ring, namely, the grouping  $[\text{CH}:\text{N}:\text{C}(\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2):\text{CH}]$  as shown in heavy lines in the formula, and that separation of parts of this essential structure by interposition of another atom (often with other groups attached to it) may be expected to convert the compound to an antagonist of histamine. Actually, this situation can be seen in some of the rather efficient antihistamines such as *Neoantergan*, *Pyribenzamine*,† *Hetramine*,‡ *Chlorothen*, for they possess in interrupted fashion features of the pharmacologically significant grouping of histamine. This argument is not readily applicable to all of the antihistamines, and, as Gaddum mentions, other approaches to an interpretation of competitive inhibition are possible.

In any event, the "antihistamines" confer definite protection, and interpretation will depend only on the degree to which the action of the protecting substances is specifically directed against histamine (Loew, 1947).

\* The arrangement of the structural formulas is reproduced from Gaddum (Gaddum, J. H., 1948, *Histamine*, Brit. Med. Jour., 1, 867-873). The respective compounds may be designated as follows:

1. 2 - isopropyl - 5 - methylphenoxyethyldiethylamine ("thymoxyethyldiethylamine")
2. N-phenyl-N'-ethyl-N'-diethylethylenediamine
3. N-benzyl-N',N'-dimethyl-N-phenyl-ethylenediamine
4. N,N - dimethyl - N'(p - methoxybenzyl) - N'( $\alpha$  - pyridyl) - ethylenediamine
5. 2 - (benzohydroxy) - N,N - dimethyl - ethylamine (" $\beta$ -dimethylaminoethyl benzhydryl ether hydrochloride")
6. N-(2-dimethylaminoisopropyl)-phenothiazine
7. N,N - dimethyl - N' - (5 - chloro - 2 - thenyl) - N'-(2 - pyridyl)-ethylenediamine
8.  $\beta$ -imidazolyethylamine

† Pyribenzamine differs in structure from *Neoantergan* only in the absence of a methoxy grouping; *Hetramine* differs from *Pyribenzamine* in the possession of 2-pyrimidyl in place of a 2-pyridyl radical.

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7

# Immunology and Immunochemistry

## INTRODUCTION

Immunology is concerned with the study of resistance to disease. In the past, this has meant largely infectious disease, but it is likely that many immunologic methods will in the future receive further application to noninfectious conditions.

Outlines of the history of immunology have been given by Topley and Wilson (1946), and by Zinsser, Enders and Fothergill (1939). Landsteiner has summarized his own extensive contributions to serology in a new edition of his monograph (Landsteiner, 1945) which also contains a comprehensive bibliography of the field. Other valuable material will be found in the books of Boyd (1947) and Sevag (1945).

In the past 25 years there have been numerous applications made to immunology of the methods of chemistry and physics, and this approach has been systematized into the science of immunochemistry—a term first applied by Arrhenius in 1907. These studies have differed from the earlier biologic investigations mainly in their attempt to define the antigens and antibodies under investigation in quantitative, molecular terms. Most of these studies have purposely been made on simplified, perhaps somewhat artificial, systems, but the conclusions are applicable to the more important problems of classic immunology. This literature has been presented in a number of reviews (Heidelberger, 1939a, b; Kabat, 1943; Treffers, 1944). Details of the experimental procedures will be found in the book of Kabat and Mayer (1948). Additional references can be obtained from the articles on immunochemistry in recent issues of the *Annual Review of Biochemistry*, and the *Annual Review of Microbiology*.

## ANTIGEN-ANTIBODY REACTIONS

### CHARACTERISTICS OF ANTIGEN-ANTIBODY REACTIONS

In each species of animal or micro-organism there are a number of substances, in particular proteins and complex carbohydrates which possess chemical groupings more or less specific in arrangement. If these substances, termed *antigens*, are introduced into an animal of a foreign species the latter endeavors to produce in its serum a substance which reacts specifically with the antigen with the object of neutralizing and removing it. The new component is termed the *specific antibody*. It is a protein which has many of the properties characteristic of the other serum globulins of the species producing it.

There are a number of methods for demonstrating antigen-antibody reactions. Their characteristics may be developed by following through a typical experiment. Suppose that a single, homogeneous protein, such as crystalline egg albumin, is selected as antigen and injected into a rabbit by an appropriate route, usually intravenously. Although antibody may often be demonstrable after a single injection, sera of high antibody content are produced only after a dozen or more injections totalling several mg. of antigen. After the last injection the animal is permitted to rest for a week or ten days to allow the antibody con-

tent to increase to a maximum, and then bled. The serum is carefully separated from the clot, clarified by centrifugation if necessary and then suitable amounts, such as 1 ml. portions, are distributed into a number of test tubes. Various amounts of the egg albumin antigen are next added, as in Table 13. The contents of the tubes are mixed and allowed to stand in the water bath at 0° C. for an hour or two.

TABLE 13. PRECIPITATION OF RABBIT ANTIBODY TO CRYSTALLINE EGG ALBUMIN BY VARIOUS AMOUNTS OF THE LATTER ANTIGEN

TUBE NUMBER	SERUM	EGG ALBUMIN ANTIGEN ADDED	APPEARANCE OF TUBES AFTER 2 HOURS AT 0° C.
	ml.	mg. N	
1	1.0	0.020	Slight precipitate
2	1.0	0.040	Moderate precipitate
3	1.0	0.080	Heavy precipitate
4	1.0	0.120	Heavy precipitate
5	1.0	0.200	Slight precipitate

The combination of antigen with the antibody contained in the serum is evidenced by a visible reaction—the formation of a specific precipitate. The latter contains both antigen and antibody. Serum from a non-immunized animal, or from an animal injected with an antigen other than egg albumin, will not give a reaction with any amount of the egg albumin antigen, a reflection of the specificity of the process.

For quantitative assay the tubes are centrifuged until the precipitates are well packed and the supernates carefully poured off and set aside for later examination. Since the reaction was carried out in whole serum, it is necessary to wash the precipitates several times with saline to free them of the large amounts of nonantibody protein. When this is done the amount of antibody in the precipitates may be determined in one of several ways. For example, the precipitates may be dried and weighed di-

rectly, although this is not convenient and is seldom done. More easily, advantage may be taken of the fact that, as antibody is a serum protein, the determinations can be made by one of the usual methods of estimating that class of substances. Of these, the micro-Kjeldahl method and the colorimetric phenol method of Folin lend themselves best to this application. If antibody is determined by the Kjeldahl method the data may be reported directly in mg. of N. If desired, this value can be converted into milligrams of antibody protein by multiplying by a suitable factor, which is 6.25 for most proteins. Table 14 presents the results of the analyses on our test system.

We have mentioned that the specific precipitate consists of antigen and antibody, without detailing the proportions of each, or whether the precipitate contains all of the antigen or antibody present in the system. To interpret the data it is necessary to have information on the latter points and we will have need of the supernates which were poured off from the precipitates after the initial centrifugations.

Each supernate is distributed between two test tubes. To one we add a minute quantity of the antigen, egg albumin. A precipitate would indicate an excess of free antibody in the supernate. To the other tube we will add a small amount of the immune serum. A precipitate here indicates an excess of antigen in the supernate added. As is evident in the last column of Table 14, the supernate of tube No. 3 yielded tests for neither antigen nor antibody, which is interpreted to mean that the amount of antigen originally chosen, 0.08 mg. egg albumin N, was just sufficient to remove all of the antibody without leaving an excess of antigen in the supernate. In practice it is found that if the amounts of antigen are spaced more closely together, more than one tube might show this behaviour, and the system is therefore spoken of as having an *equivalence zone* rather than an *equivalence point*.

The data in Table 14 may be plotted, as



TABLE 14. TOTAL PROTEIN AND ANTIBODY PROTEIN PRECIPITATED, AS DETERMINED BY THE MICRO-KJELDAHL ANALYSES \*

TUBE NUMBER	SERUM	EGG ALBUMIN ANTIGEN ADDED	TOTAL PROTEIN PRECIPITATED	ANTIBODY PROTEIN PRECIPITATED †	TEST ON SUPERNATE
	ml.	mg. N	mg. N	mg. N	
1	1.0	0.020	0.303	0.283	Excess antibody only
2	1.0	0.040	0.539	0.499	Excess antibody only
3	1.0	0.080	0.813	0.733	Neither antibody nor antigen
4	1.0	0.120 (only 0.087 precipitated)	0.730	0.643	Slight excess antigen
5	1.0	0.200 (only 0.048 precipitated)	0.414	0.366	Large excess antigen

\* Modified, by interpolation, from Heidelberger, M., and Kendall, F. E., 1935, A quantitative theory of the precipitin reaction. III. The reaction between crystalline egg albumin and its homologous antibody. Journal of Experimental Medicine, 62, 697-720.

† By difference between total protein N precipitated and antigen N precipitated.

in Chart 3. Curve A represents the experimental findings, the amount of precipitate obtained (in mg. of N) for each amount of

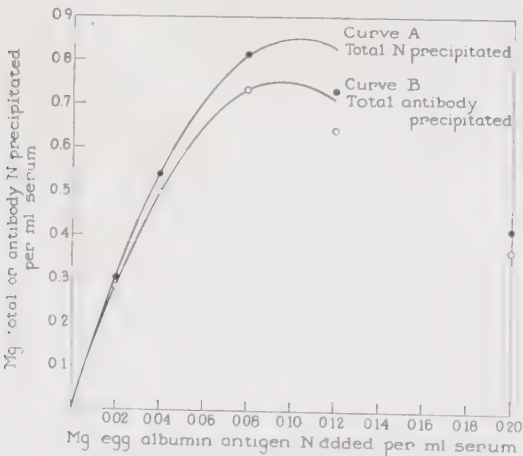


CHART 3. Total nitrogen and antibody nitrogen precipitated by Egg Albumin antigen from an immune rabbit serum. The circles are experimental; the curves are calculated from equations discussed in the text (p. 167).

antigen used. If the antigen had been a non-nitrogenous substance, such as certain bacterial polysaccharides, curve A would also represent the amount of antibody precipi-

tated, for, although the precipitate contains both antibody and antigen, the latter would not register by the Kjeldahl technic. In the example which we have used, however, the precipitate contains antigen N as well, and this must be deducted from the total N so that the antibody N will be given by the difference. It is a valuable characteristic of systems containing only a single antigen that in the region of excess antibody (such as tubes No. 1 and No. 2 of Table 14) and in the equivalence zone (which includes our tube No. 3) all of the antigen added is precipitated by the antibody which comes down. In the region of antigen excess (tubes No. 4 and No. 5), although the total N curve accurately expresses an experimental fact, the antibody N curve cannot be obtained from it by direct subtraction, since only a portion of the added antigen is precipitated, while the rest remains in the supernate. As will be described shortly, it is possible to analyze for the amount of antigen not precipitated, and thus to determine the amount which did precipitate, from which the correction can be made as before.

The amount of antibody precipitated de-

creases as larger and larger amounts of antigen are added beyond the equivalence amount, and if sufficiently large amounts of antigen are used there will be complete inhibition of precipitation. In this region (the inhibition zone) antigen and antibody combine to form soluble complexes. Most antigen-antibody systems are not too sensitive to slight excesses of antigen, which indeed may be necessary to remove the last trace of antibody. Under these conditions the antibody curve (corresponding to B, Chart 3) exhibits a flat plateau before descending. Although a single analysis would not enable one to know whether the ascending or descending portions of the curve were being dealt with, this fact could readily be ascertained by examining the supernate for excess antibody or antigen.

The top of the antibody curve represents the maximum amount of antibody precipitable under the conditions chosen and is therefore a direct and objective measure of the content of this particular antibody in the serum.

So far, nothing has been said about the rate at which antigen-antibody reactions proceed. A careful distinction should be made between the rate of the reaction (as judged by the time of appearance of turbidity or flocculation) and the amount of precipitate which can eventually be obtained, since these two are by no means directly related. Competition experiments (Mayer and Heidelberger, 1942) have demonstrated that the initial combination of antigen and antibody may be extremely rapid, a matter of a few seconds, but that the subsequent aggregation to give a visible flocculation may be slow and require many hours. The rate of appearance of a precipitate is markedly influenced by the volume of the system, being slower as the reactants are diluted. Except for very slight solubility corrections, the amount of antibody precipitated depends only on the amount of antigen added to a given amount of serum and not on the total volume of the system.

The same applies for the effect of temperature on many antigen-antibody reactions. These proceed slowly at 0° C., and much more rapidly at elevated temperatures, 37° to 56° C. If sufficient time is allowed, however, it is often found that the quantity of antibody precipitated in the cold is 20 per cent or more greater than that precipitated at the higher temperatures. This is a factor of obvious importance if the true antibody content of the serum is desired.

#### QUANTITATIVE ABSOLUTE METHODS

The data of Tables 13 and 14, and Chart 3 are examples of a system studied by quantitative absolute methods. First presented by Wu in 1927, these have been greatly extended by Heidelberger, Kendall, Kabat, and their associates (see reviews of Heidelberger, 1939a, b). The essential features are that the reactions are carried out under the conditions of analytical chemistry, with accurately measured volumes of antigen solutions and of serum delivered from calibrated pipettes, and subsequent determination of the antibody precipitated by an appropriate analytical procedure. If the analyses are made by the micro-Kjeldahl method, duplicates will usually agree to within 0.02 mg. N, which will constitute an uncertainty of from 2 to 4 per cent at the optimum sample levels of 0.5 to 1.0 mg. N. The greatest disadvantage is that if the sera are too weak this amount of antibody may not be available in any convenient working volume. The methods are not limited to soluble antigens; insoluble particles such as bacteria, (agglutinin reaction), specific precipitates (Treffers et al., 1942) or erythrocyte stromata (Heidelberger and Treffers, 1942) may be used. With the insoluble antigens there is no danger of running into the inhibition zone of antigen excess.

For the analysis of smaller amounts of antibody, as in human sera, protein may be determined colorimetrically (Heidel-



berger and MacPherson, 1943; Heidelberger et al., 1946). In dealing with very weak sera it is important that sufficient time be allowed for the precipitate to form; 7 to 10 days at 0° C. may be necessary. If fresh human or other sera are being examined it is also important to take into account the presence of complement in such systems since this will add to specific precipitates and may well double their analytical "weight." Traditionally, complement has been removed by inactivation through short periods of heating to 56° C. It has been shown, however, that this results in considerable loss of antibody, and other procedures are to be preferred (Heidelberger et al., 1946).

Rapid turbidimetric procedures have been described which may be read after reaction periods as short as 30 minutes. Although they yield data in arbitrary instrumental units, the curves are of the same general form as those obtained with the micro-Kjeldahl method and can be converted to absolute units by one or more simultaneous Kjeldahl analyses (Libby, 1938a, b; Goebel, et al., 1945).

Although these quantitative methods are more frequently used for the determination of antibody, they may be used equally well to determine antigen, for example, that remaining free in the inhibition zone. It will be recalled that data of this type were necessary for the complete solution of the problem presented in Table 14. A curve such as A (Chart 3) represents the total amount of precipitate given by a known amount of antigen. Once the serum has been calibrated in this manner, new portions of it can be set up against unknown amounts of antigen, and from the total amount of precipitate obtained the corresponding amount of antigen can be found by reference to the standard curve. This method of analysis can assay to within a few per cent as little as a microgram of antigen contained in a mixture of thousands of times its weight of serologically

unrelated protein and polysaccharide. Among the other applications of this procedure are the determination of particular proteins in serum (Goettsch and Kendall, 1935; Kendall, 1937) and the amount of host tissue fraction contained in influenza virus (Knight, 1946).

In addition to providing objective, absolute figures which can be reproduced by every laboratory within the limits indicated, these methods have proven indispensable for studies on antigens, antibodies and the mechanisms of their interaction. For example, it is only by knowing the absolute amounts of antibody reacting with an antigen that the molecular ratios of the two in specific precipitates can be calculated. Data of this sort have helped to eliminate serologic reactions as vague colloidal phenomena and to place them in the framework of definite chemical reactions which conform to well-known laws. Consideration of these will be postponed until after the other available serologic reactions have been examined.

#### ABSOLUTE VS. RELATIVE MEASUREMENTS

Serologic procedures may be divided into two kinds, those giving absolute data, i.e., which measure antibody or antigen in terms of milligrams or other weight units available in every laboratory, and those giving only relative data, in which one antibody or antigen is estimated to be so many times stronger than another. These differences in basic principle should not be confused with differences in accuracy, that is, the degree to which valid differences can be measured, since this latter point depends largely on whether visual or instrumental means are used. In theory, both general procedures could employ accurate instrumental methods. In practice, however, the absolute methods depending on chemical or instrumental procedures will permit antibody estimations to within 5 per cent or less, while available relative titer methods based

on visual endpoints seldom permit estimations of relative antibody contents better than to twofold dilutions or 100 per cent.

The precision obtainable with absolute serologic methods may be desirable or even absolutely necessary if conclusions are to be made from certain types of data, as will be illustrated below. However, for many diagnostic purposes this precision is not necessary, and relative titer methods may be quite sufficient. In using the latter it is nevertheless important that the experimenter be aware of the fundamental limitation of relative methods.

In order to read the endpoint in a typical relative serologic titration it is necessary that there be sufficient antibody to give a visible reaction. Thus, in determining the agglutination titer (p. 161) the greatest dilution of serum which will cause visible agglutination of a bacterial suspension is taken as the endpoint. It can easily be demonstrated that different species of bacteria require different amounts (i.e., milligrams or other weight units) of antibody to give this limiting visible agglutination. Even the same strain may require different absolute amounts, depending on whether the agglutination is due to antibody directed against the flagellar or the somatic antigens. Flagellar agglutination appears to require very little antibody, and, hence, typical antisera such as that to the typhoid flagella (H antibody) give titers running into the thousands. On the other hand, agglutination of the typhoid organism by antisera specific for the bacillary bodies (O antibody) requires relatively more antibody to produce a visible effect with the result that the apparent titer is lower, usually in the hundreds.

Titer methods give a measure of the relative amounts of antibody contained in various samples of sera as long as only one serologic system—a particular antigen and its specific antibody—is dealt with, since the intrinsic amount of antibody required for the endpoint will then be con-

stant throughout. The limitation of relative methods begins when it is desired to compare the amounts of antibody present in sera to two different antigens, since there is no way of determining the sensitivity of each system without resort to an absolute measurement on each.

The situation might be likened to determining how far a vehicle has travelled from a knowledge of the gasoline consumed. Thus, given a fleet of trucks averaging 5 miles to the gallon, one could estimate quite closely their individual mileages from the records of the gasoline consumed by each. One could do the same for a fleet of motorcycles, averaging 35 miles to the gallon. However, one could not make any prediction as to the mileage from the gasoline record of a vehicle selected at random without knowing whether it was a truck or a motorcycle, nor compare the relative mileages of two vehicles from this data without knowing whether they were both of the same type. Neglect of this point, in the serologic analogy, has led to the advancement of many erroneous statements concerning relative antigenicities.

#### PRECIPITATION PROCEDURES

The simplest method for demonstrating the presence of antibody consists in carefully layering a fairly concentrated solution of antigen over serum contained in a narrow test tube. The serum, being heavier, will remain at the bottom, and a sharp boundary will be formed. As the two reagents diffuse into one another a thin ring of precipitate will develop in the layer where optimal conditions for precipitation prevail (ring test). This procedure is most useful when no data as to the content of antigen or antibody are available and when it is desirable to economize material without wasting dilutions in a possible inhibition zone. It is rapid and easily carried out, but the results are of qualitative significance only. It is nevertheless a valuable tool where only an indication of the presence or absence of antigen or antibody is required.

One of the classic serologic procedures is



the *precipitin titration*. This consists in making dilutions of antiserum (generally twofold) and adding to each a constant amount of antigen. The greatest dilution of serum which still gives a visible precipitate is called the precipitin titer. Alternatively, the amount of antiserum may be kept constant and the dilutions of antigen varied, but unless the amount of precipitate is measured by one of the quantitative absolute methods, this procedure is not as sensitive to differences in antibody contents as is the former, in which antibody was the variable. Some antigens such as bacterial carbohydrates may give visible precipitates at a dilution of 1:10,000,000, so that the test is a sensitive one. In spite of this (or perhaps because of it) the reaction is difficult to quantitate better than to a twofold dilution or 100 per cent.

A somewhat better variation of the precipitin titration consists in preparing dilutions of either antibody or antigen (the other being kept constant) and noting the tube in which precipitation is first evident (optimal proportions method). This procedure is based on the fact that the rate at which the antigen-antibody mixtures flocculate depends, among other things, on the relative proportions in which the reactants are present. This is not surprising since antigen and antibody combine in a continuously variable series of proportions determined by their relative amounts (p. 167) and each proportion has a characteristic consistency, solubility, etc.

If undiluted serum is placed in each of a series of tubes to which decreasing amounts of antigen are added it is frequently observed that flocculation occurs first in the tube to which an equivalence amount of antigen was added so that the supernatant contains neither antigen nor antibody. Greater or lesser amounts of antigen result in slower appearance of precipitation, the time depending roughly on the position away from the equivalence tube. If the serum is now diluted 1:4 and the experiment repeated,

it is found in consequence of this dilution that the tubes as a group flocculate more slowly than before but it still remains true that for all mixtures of this serum with antigen the new equivalence tube will cloud first. In this second series the equivalence tube will contain only one quarter of the amount of antigen needed to neutralize the undiluted serum in the first series. The rationale will thus be apparent: the optimal proportions method is a means of identifying antigen-antibody mixtures of some constant combining proportion. As has been stated, this is most frequently a mixture in the equivalence zone, but serologic systems are known in which the most rapid flocculation occurs with slight excess of antibody or in others, of antigen.

For a series of sera of the same qualitative specificity the amounts of antigen necessary to give some definite combining ratio with antibody are directly proportional to the relative antibody contents of the sera. The foregoing considerations apply strictly only in the case where a single antigen reacts with its own antibody, but this reservation has frequently been ignored. A comprehensive critical discussion of the reaction, including a comparison of the constant antigen and constant antibody procedures has been given by Boyd (1941).

#### AGGLUTINATION REACTIONS

Although most bacterial antigens can be reduced to soluble forms, it is often more convenient to work directly with the particulate state. For most purposes the antigen may be regarded as a potentially soluble substance which is firmly attached to the bacterium by a chemical bond. It can still combine with antibody, which acts as a bridge linking the organisms together in clumps (agglutination reaction). As with the precipitin reaction, this type of agglutination is specifically determined by the antigens and antibodies involved. Titrations

may be carried out with either living or dead bacteria by diluting out the serum and observing the titer at which agglutination is just evident. The reaction is facilitated by heat (from 37° to 56° C.) and by stirring, which is sometimes effected by immersing the tubes only part way into the bath so that convection may take place.

The reactions may also be carried out by the quantitative absolute procedures similar to those employed above for the precipitin reaction. The antigen is added in the form of a washed bacterial suspension of known N content and the antibody is determined from the N recovered in excess over that added. (Heidelberger and Kabat, 1937).

In evaluating the data the multiple nature of bacterial antigens must be kept in mind, since bacteria may be agglutinated by antibodies to many of the antigens contained in the cell. Flagellated bacteria such as the *Salmonella* may be agglutinated in distinctly different ways, involving either one of the flagellar antigens or the somatic antigens. Sometimes one antigen may be masked by the presence of others; if the latter are thermolabile, as appears to be the case in certain dysentery and coliform bacilli, the hidden antigen may be made reactive by boiling the suspension.

The agglutination reaction has important diagnostic applications. It may be used to identify the serologic types of bacteria isolated from cases, or it may be used to detect the presence of antibody in the patient's serum to confirm recent or present contact with the etiologic agent, as in typhoid fever or brucellosis. Where antibodies occur in the absence of acute infection, due to previous vaccination or the persistence of antibodies after convalescence, estimations of the antibody titer must be made, preferably at more than one time, to determine whether the titer is rising or falling. Where common group antigens exist it is important that the sera be properly absorbed before specific typing is attempted.

#### ABSORPTION REACTIONS

If a serum contains antibodies to more than one antigen it may be rendered specific for one of these by removing the others. For example, bacteria of one general group usually contain enough antigens in common so that organisms of one serologic type will give some reaction with antisera to any of the other types. If a serum to one type is therefore permitted to react with one or more other serologic types of organisms of that group the common antibodies can be removed and the supernate will contain only antibody for the homologous type, specific for the organism originally injected. Although absorptions are more conveniently performed with particulate antigens such as bacteria or blood cells, they can be done with soluble antigens as well. In the latter case, however, care must be exercised that not too large an excess of absorbing antigen is used since it may then begin to inhibit antibody. When properly used, absorption technics greatly broaden the range of serologic methods, both by preventing false readings due to common antigens and by permitting separation of two antigens which could not otherwise be distinguished readily. By their use, Irwin (1947) has identified over 20 different antigens in hybrid species of birds.

#### COMPLEMENT

One manifestation of complement activity—the ability of freshly shed blood to resist putrefaction for some time—was noted by John Hunter in 1792. The principles underlying this process have been the subject of much experimental attention, commencing with the work of Nuttall, Bordet and Ehrlich at the end of the last and the beginning of this century. The most valuable result was the demonstration that there were many factors involved in complement action, and the empirical standardization of these for various diagnostic



applications. In recent years a considerable clarification of the factors has been achieved, but a comprehensive theory of their mechanism of action is still lacking. The basic properties have been reviewed by Zinsser et al. (1939), and the more recent developments by Pillemer (1943) and Heidelberger (1946).

Complement is the name given to a group of proteins found in normal serum. As listed by Pillemer, the properties of complement include the following: the hemolysis of sensitized (i.e., antibody-coated) red blood cells; the lysis of certain sensitized bacteria; the capacity to kill sensitized bacteria in the absence of bacteriolysis; the opsonization (pp. 80, 100) of sensitized bacteria in the absence of immune serum; the activation of thermostable opsonins; the alteration of the rate of aggregation of antigens by their homologous antisera; the alteration of the state of aggregation of antigen-antibody systems; and finally the property of combining with most antigen-antibody systems even in the absence of any visible manifestations (complement fixation). Complement may also be involved in the destruction of viruses, changes in sedimentation rates of red blood cells and other processes.

At present four distinct components of complement are known. These are designated as C'1, C'2, C'3 and C'4 [the prime is added to avoid confusion with bacterial group-specific C substances].

The chemical and physical properties of three of the four components have been determined (Table 15). Component 3 (C'3) appears to be a lipoprotein but has not yet been isolated in a sufficiently homogeneous state to permit determination of its properties. All the components are globulins and the ones listed contain appreciable amounts of carbohydrate. The various components were first identified on the basis of their inactivation by different treatments, the activities ascribed to C'2 and C'4 appear to be associated with a single chemical molecule.

Until very recently complement was determined only on a relative titer basis. Through the experiments of Ecker and Pillemer, and

TABLE 15. PROPERTIES OF PURIFIED COMPONENTS OF COMPLEMENT FROM GUINEA-PIG SERUM \*

	TYPE OF PROTEIN	
	EUGLOBULIN	MUCO-EUGLOBULIN
Precipitated by (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> of concentration . . . . .	1.39 M	2.2 M
Complement components present . . . . .	1	2 and 4
Electrophoretic mobility (phosphate buffer, ionic strength 0.2, pH 7.7) × 10 <sup>5</sup> . . . . .	2.9	4.2
Sedimentation constant, s <sub>20</sub> × 10 <sup>13</sup> . . . . .	6.4	
Carbohydrate content, per cent. . . . .	2.7	10.3
Optical rotation, [α] <sub>D</sub> . . . .	−29°	−193°
Destroyed in 30 min. at °C. . . . .	50	50 (comp. 2) 66 (comp. 4)
Fraction of total serum protein, per cent. . . . .	0.6	0.2

\* For further details, the review by Pillemer (1943) should be consulted.

Heidelberger and Mayer, and their collaborators, it has been possible to determine the absolute amounts contained in human and guinea-pig sera, either by direct isolation or by quantitative assay of the amounts added to specific precipitates. This data will probably be of increasing importance for studies on the mechanism of complement action.

It is not known whether all components of complement participate in every one of the reactions listed above; this point may be clarified with tests employing the purified components. It is known, however, that all components are necessary for the hemolysis of sensitized red cells and for bactericidal action (Dozois, Seifter and Ecker, 1943; Seifter, Dozois and Ecker, 1944).

Although an excess of some components will partially compensate for a deficiency in one component, it was shown in 1938 by Hegedus and Greiner that when a serum containing complement is diluted out for an end-point test the titer is determined by the component present in least amount. Titrations of the relative amounts of any individual components in samples of sera can be made by ensuring that there is an excess of the other

three, so that the desired component becomes the limiting factor. The details and numerous precautions have been given by Bier et al. (1945). Corresponding components from human and guinea pig complements can be used interchangeably, i.e., human C'1 will function in the presence of guinea-pig C'2, 3 and 4.

It follows that the highest complement titers will not necessarily be given by those sera which contain the largest amounts of one or more components but by those sera which have the largest balanced amounts of each component. It is for this reason that guinea-pig serum provides the largest amount of usable complement, as has long been known from practical experience. Human or bovine complements contain relatively large amounts of one or more components, but they have such a low limiting titer of one or more others that the net titer is small. Some of the complement components are quite unstable and deteriorate rapidly on standing or if they are heated.

**C'1.** This protein constitutes about 0.6 per cent of the total protein (0.03-.05 mg. N) in human or guinea pig serum. It is heat labile and also relatively unstable on standing at neutral pH although it may be preserved for some time between pH 5.4 and 6.0 C'1 appears to constitute the largest component by weight which adds to specific precipitates. The titer in human serum approximates a dilution of 1:4000, in guinea pig serum, 1:2500. C'1 exhibits its full activity at protein concentrations of from 0.002 to 0.02 per cent but is inhibited at higher protein concentrations. It is the largest component in sheep, horse and bovine complements, which, however, give low net titers since they lack almost entirely the other components.

**C'2.** This activity appears to be associated with the same molecule which carries C'4 activity. The latter can be distinguished by its relative heat stability: heating to 56° C. for 30 minutes destroys C'2 activity but leaves C'4 activity. The yield from guinea pig serum is about 0.2 per cent of the total serum protein. C'2 is generally the limiting component in human complement.

**C'3.** Although C'3 is relatively resistant to inactivation at 56° C. it is the first component to disappear on standing, even at room

temperature. This component was discovered through the observation that washed yeast cells would eliminate the complement activity of fresh guinea-pig serum. An insoluble carbohydrate has since been isolated from yeast which carries this property; quantitative experiments indicate that the carbohydrate can remove 0.02-.03 mg. N per ml. serum, although it is not certain that all this represents C'3. This component is generally the limiting factor in guinea pig complement. The anticomplementary activity (p. 165) of bacteria may be due largely to their specific removal of C'3.

**C'4.** Complement may also be rendered inactive by treatment of the serum with ammonia. This is a specific property of all primary amines and is not due to a pH effect. That the component destroyed is not identical with the others may be demonstrated, for example, by mixing it with yeast-inactivated complement (which lacks C'3). Full activity is restored. As has been mentioned, C'4 activity is relatively heat stable and is associated chemically with C'2 activity.

**Midpiece and endpiece** are frequently used to designate the fractions first obtained by Ferrata in 1907 by dialysis of guinea pig serum against distilled water. Similar preparations are used in the titration of individual components. Neither the redissolved precipitate nor the supernatant from the dialysis exhibits complement activity, but if they are mixed almost full activity is regained. The water-insoluble precipitate is now known to contain all the C'1 and the major part of the C'3 activity. The term "midpiece" has been applied because it was believed that fixation of this component occurred before that of the other, or "endpiece." The latter, representing the water-soluble components, contains C'2 and C'4 activities.

#### IMMUNE HEMOLYSINS

Bordet observed in 1909 that if rabbit erythrocytes were injected into guinea pigs the serum of the latter acquired the power of agglutination, and if freshly drawn, of lysing the introduced cells. The lytic property disappeared if the serum was heated at from 50° to 60° C. but could be restored if fresh normal serum were added. The heat-stable component will be recognized as anti-



body specific for the cells, the heat-labile component as complement, present in normal as well as immune sera.

Immune hemolysins, or in Ehrlich's terminology, amboceptors, are antibodies which will lyse cells in the presence of complement. Blood cells which have been coated with this antibody are termed "sensitized cells." They play an important role in experiments employing complement since they act as indicators for its presence. If sufficient complement is present complete lysis of the sensitized cells will occur; less complement will result in partial lysis, and the complete absence of complement in no lysis. Conversely, complement and cells may be used as indicators for the presence of hemolysin. In such cases control tubes containing only cells and complement must be used, as guinea pig sera may contain sufficient normal antibodies to the cells to furnish the necessary hemolysin without addition of any other material.

The units of hemolysin and of complement are generally defined in terms of the greatest dilution of serum which will just cause complete lysis of blood cells in the presence of an excess of the other factor, complement or hemolysin, respectively. The volume, amount of cells, temperature and time of reading must of course be specified. One, two, four or more units may be employed in a given experiment, depending on how large a known excess is desired. A plot of the degree of lysis of sensitized cells against the amount of complement added is essentially a distribution curve of cell resistance or fragility. It is well known that curves of this type are best studied not by determining the position of the hardiest survivor (corresponding to incipient complete lysis) but by determining the position of the midpoint (or 50 per cent lysis). Analogous criteria are now widely used in the study of animal resistance to infectious or toxic agents ( $LD_{50}$  instead of M.L.D.).

Complement titrations employing 50 per cent hemolysis endpoints, as determined by visual comparison with standards, have been applied to the diagnosis of syphilis and other diseases (Wadsworth, 1939). The use of photo-electric colorimeters has permitted more precise determination of such endpoints (Mayer et al., 1946; Kent et al., 1946). Although at first sight somewhat formidable, these methods may actually be simpler in practice than titrations employing complete lysis endpoints in that fewer tubes of dilutions need be set up. It is also possible to compare, for an average value, readings obtained on two or more tubes instead of the single reading afforded by the complete lysis endpoint.

The phenomenon of hemolysis has been extensively studied. From the data on the absolute amounts of complement and antibody involved in a typical hemolytic experiment, it can be calculated that as few as 500 molecules of rabbit antibody need be attached to an erythrocyte before lysis and that from only 0.01 to 0.3 per cent of the cell surface is then covered (Heidelberger et al., 1941). Whatever the nature of the lytic process, it apparently involves a relatively small and presumably sensitive area of the cell.

Mayer, et al. (1946) have recently demonstrated that  $Mg^{++}$ , or other divalent ions such as  $Ca^{++}$ ,  $Ni^{++}$  or  $Co^{++}$  are essential for the hemolytic action of complement. In addition to the possible implications as to an enzymatic mechanism involving an ionic cofactor, this study demonstrated that complement titrations are frequently carried out under suboptimal concentrations of these ions, which then become limiting factors instead of the components which it is desired to titrate. It also provides an explanation for the anticomplementary activity of certain substances, such as citrate, oxalate or phosphate which bind and remove  $Mg^{++}$ .

## COMPLEMENT FIXATION

Complement is taken up by specific precipitates, a property utilized by Bordet and Gengou in 1901 for the development of diagnostic tests. The principle is quite simple. Suppose a solution of a known antigen, A, and an antiserum to be examined for anti-A. The two are permitted to react in the presence of complement. If there is actually antibody to A in the serum its reaction with antigen A will bind to it some or all the complement present. This removal of complement may be determined by adding an indicator system—sensitized red cells—and incubating at 37° C. for a short time. If the complement has been bound by the antigen-antibody reaction there will not be enough left to effect complete hemolysis of the sensitized cells. The absence of lysis will thus denote an antigen-antibody reaction from which it may be concluded that antibody must have been present. On the other hand, complete lysis of the cells would mean free complement, and therefore no antibody to bind it. Although the complement fixation reaction is generally used to detect the presence of antibody, it works equally well in reverse. If a known antibody is supplied it will detect the presence of antigen.

The reaction is exceedingly delicate. In one quantitative series which employed the more insensitive complete-hemolysis endpoint, 0.04  $\mu$ g. antibody N, or 0.01  $\mu$ g. antigen (pneumococcus polysaccharide) could easily be detected. At these high dilutions the solutions remain crystal clear and give no visible evidence of a precipitate. The complement removed from solution may "weigh" as much (in terms of N) as the antigen-antibody precipitate.

Although the complement fixation reaction is generally applicable to a wide variety of antigen-antibody systems, it should be noted that certain horse sera to bacterial and other antigens do not fix complement,

although rabbit and bovine antisera to these antigens give strong fixation. On the other hand, a good many antigens, or sera, may by themselves fix complement before addition of the corresponding antibody or antigen. This is known as *anticomplementary action*, and must be guarded against by proper control tubes. It may be overcome in most instances by dilution or heating. Strict attention to detail is essential for the proper conduct of complement fixation reactions, and every reagent must be properly controlled. The necessary precautions are discussed in the papers just referred to, and in standard texts on laboratory procedure.

## WASSERMANN REACTION

Properly controlled complement fixation reactions exhibit the same high specificity as other antigen-antibody reactions. Paradoxically, however, the most widely used application of the complement fixation reaction—the familiar Wassermann test for syphilis—is in part a nonspecific reaction. As first conceived in 1906 by Wassermann and his associates, it was a logical extension of the Bordet-Gengou reaction just described. An extract of the liver of a syphilitic fetus was employed as antigen to test for antibodies in sera from suspected cases of syphilis. As was hoped for, sera from known cases resulted in complement fixation and sera from uninfected persons generally gave a negative test. In fact, further investigation of some of the positive sera in the latter group showed that these came from hitherto unsuspected cases. The reaction therefore appeared to be a specific one. It was soon found, however, that aqueous, or better, alcoholic extracts of normal livers served equally well as "antigen." The reaction is therefore nonspecific to the extent that the antigen need not be prepared from syphilitic material; at present, an alcoholic extract of normal heart muscle, fortified with cholesterol, is generally



used. The reaction does exhibit a high degree of specificity in a diagnostic sense in that a positive reaction (complement fixation—no hemolysis of the indicator cells) is highly presumptive evidence of the presence of syphilitic antibody. A small percentage of sera, including those taken in certain infectious diseases such as malaria and leprosy give false positive Wassermann reactions but a discussion of these is outside of our scope. References to the nature and specificity of the reaction will be found in the papers of Weil (1941), and Davis (1944).

#### BACTERICIDAL TESTS

Pfeiffer observed in 1894 that cholera vibrios injected into a guinea pig previously infected with these vibrios showed marked morphologic changes and soon disappeared by lysis. Examination of the factors responsible revealed a relatively thermostable component, specific antibody, and a thermolabile component, complement. The situation therefore paralleled that of immune hemolysis which was, however, discovered later as a result of further investigations on this so-called Pfeiffer phenomenon—the lytic action of antibody and complement on micro-organisms.

Although most Gram-negative organisms are killed by specific antibody in the presence of complement, they are not all visibly lysed, and a distinction is therefore made between bactericidal and bacteriolytic properties. As a rule only the number of viable organisms remaining after various reaction times are determined by plating, and it may be difficult to distinguish experimentally between the two mechanisms. An excess of immune serum has frequently been reported to inhibit the reaction (Neisser-Wechsberg phenomenon). Although laboratory evidence suggests that immune bactericidal and bacteriolytic phenomena play an important role in resistance to infection with Gram-negative organisms, clinical evidence to that

effect is still lacking (Zinsser et al., 1939). Gram-positive organisms are very resistant to killing by these means.

A number of other biologic tests described in greater detail elsewhere are also used to detect the presence of antibody or of antigen. Thus, the neutralization test consists in determining whether the infectious properties of a living agent, or the toxic properties of a toxin are neutralized by serum. The latter may be incubated with the agent or toxin before injection, or it may be injected separately into the animal, either before or after the substances it is sought to neutralize. Protection tests may be carried out by challenging an animal which has been actively immunized, or one which has received, passively, serum from a second animal, as in the neutralization test. Anaphylactic reactions (pp. 114, 116) and skin tests (pp. 127-129) are also used.

#### TYPES OF ANTIBODY

As each of the tests discussed above was discovered a separate name was applied to the antibodies concerned. Mention is therefore made of precipitins, agglutinins, complement-fixing or protective antibodies, etc. It was appreciated quite early that the activities displayed in the various tests might all be properties of a single antibody. It was shown, for example, that the removal of a precipitin might simultaneously take out agglutinins or other antibodies. The most rigid proof has come from experiments employing purified antibodies to single antigens.

Although there is thus good evidence that an antibody to a single antigen may exhibit all of the usual activities, it cannot be assumed without further proof that any given antibody must necessarily do so. Not all antibodies will fix complement in the presence of their antigens. So-called soluble, univalent or incomplete antibodies (p. 185) are known which will precipitate with antigen only under very restricted conditions.

On the other hand, neutralizing properties are frequently ascribed to sera when no other serologic reaction can be demonstrated. Although the existence of antibodies with limited serologic activities cannot be denied, consideration should be given to the quantitative aspects before this explanation is invoked in new instances. Certain reactions such as complement fixation and neutralization are so sensitive that the amount of antibody required may be well below the limits at which the precipitin reaction can be demonstrated. Unless, therefore, it can be shown that there is a sufficient absolute amount of antibody for a precipitin reaction to be expected if the antibody were of the usual type, the designation of the antibody as atypical carries little weight. It should be evident from the discussions in earlier sections that complement fixation or neutralization titers, no matter how large, do not provide information on the absolute amounts of antibody since they include unknown constants of sensitivity whose values depend on the particular system.

#### QUANTITATIVE ASPECTS OF ANTIGEN-ANTIBODY REACTIONS

Antigens and antibodies combine in multiple proportions which depend on the relative quantities of the reactants. This can readily be verified from Table 14. Thus, in tube No. 1, 0.283 milligrams of antibody N was precipitated by 0.020 milligrams of antigen N; the ratio of antibody to antigen for this first experimental point in the region of antibody excess is therefore .283/.020 or 14.2. Similar calculations give ratios of 13.0 for tube No. 2, also in the region of antibody excess, 9.2 for tube No. 3 in the equivalence zone, and 7.4 and 7.8 for tubes No. 4 and No. 5 in the region of antigen excess. For the latter, the antigen values taken are the amounts actually precipitated, not those added. Due to various uncertainties, data in this region are not

given the same quantitative significance as are the figures obtained in the region of antibody excess, and the equivalence zone.

The course of the precipitin reaction as given by the antibody N curve B (Chart 3) can frequently be represented as far as the equivalence zone by the equation

$$1 \quad \text{antibody N precipitated} \\ = k_1 (\text{antigen}) - k_2 (\text{antigen})^2$$

where (antigen) represents the amount of antigen added and precipitated, and  $k_1$  and  $k_2$  are constants. If this equation is divided through by (antigen) there results

$$2 \quad \frac{\text{antibody N precipitated}}{(\text{antigen})} \\ = k_1 - k_2 (\text{antigen})$$

which is the equation of a straight line. Taking again the data of Table 14, if the antibody:antigen ratio of 14.2 is plotted against the amount of antigen used, 0.020 mg. N, and the same is done for each of the other experimental values, the points will be seen to fall on a straight line. The latter is then continued until it intersects the ordinate (y-axis). As has been noted above, the smaller the amount of antigen added, the larger the ratio of antibody:antigen precipitated. The intersection of our line with the ordinate axis therefore gives the limiting ratio when the amount of antigen is decreased to zero. It represents the maximum amount of antibody which can be taken up by one antigen weight unit (here 1 milligram of antigen N). It can be approached but never realized, experimentally, by employing smaller and smaller amounts of antigen. As the reader may verify by plotting the data, this limiting ratio in our example is 15.8. It is the constant  $k_1$  required for Equation 2 above. This is a quantitative characteristic of the serum and does not depend on the total amount of antibody present but on its combining affinities. These will vary with the antigen used, the species immunized, etc. If the animal from which the serum was drawn receives further courses of injections the constant  $k_1$  may increase for the later bleedings (Heidelberger and Kendall, 1935; Heidelberger, Treffers and Mayer, 1940). This rise may be interpreted to mean that on continued immunization new groupings on the antigen become active in eliciting new forms of antibody which can be accommodated on



the antigen. This is analogous to the well-known broadening of the specificity of sera with continued immunization.

From our plot, the slope of the line is seen to be  $-83$ , which is the value of the constant  $k_2$  required for Equation 2. On substituting these two numerical values into Equation 1, the data of Table 14, and the curve B (Chart 3) can be expressed as

mg. antibody N precipitated

$$= 15.8 (\text{antigen}) - 83 (\text{antigen})^2$$

It was these values for the constants which were used in plotting curve B for Chart 3. The correctness of this equation can also be checked by substituting any desired value of antigen, for example, the 0.020 mg. used in the first tube, to give  $15.8(.02) - 83(.02)^2 = 0.283$  mg. antibody N precipitated, in excellent agreement with that found experimentally. Of course, perfect agreement with all values cannot be expected in an actual experiment. Nevertheless, the average deviation between the calculated and the experimental values for the 9 points listed in the original (Heidelberger and Kendall, 1935) is only 2 per cent, with individual deviations of from 0 to 8 per cent.

When plotted according to Equation 2 the data for some antigen-antibody systems do not lie on a straight line but on a more or less pronounced curve, which makes extrapolation for the limiting ratio difficult. Most of these exceptions can be fitted, however, by the empirical expression:

3 mg. antibody N precipitated

$$= k_1 (\text{antigen}) - k_2 (\text{antigen})^3$$

This is also converted to the linear form by division:

4  $\frac{\text{antibody N precipitated}}{(\text{antigen})}$

$$= k_1 - k_2 (\text{antigen})^{1/2}$$

These equations are employed exactly as are Equations 1 and 2 above except that to obtain the constants the ratios of antibody: antigen must be plotted against the square root of the amounts of antigen employed. The numerical values of the constants will depend on which set of equations is used. Thus, by Equation 3, the data for Table 14 now become

mg. antibody N precipitated

$$= 19.4 (\text{antigen}) - 36 (\text{antigen})^{3/2}$$

and by substituting a value for antigen, such as 0.020 mg., we obtain

mg. antibody N precipitated

$$= 19.4 (.020) - 36 (.020)^{3/2} = 0.288$$

which is again in agreement with experiment. This particular system is somewhat exceptional since it appears to be equally well described by both equations; the average deviations of the values predicted by Equation 3 are also 2 per cent.

These equations have several interesting and useful properties:

(1) It takes in principle only two experimental points to determine the straight line demanded for Equation 2 or Equation 4, although three are better, to verify that the data do indeed fall on such a straight line unless this is known to be the case for the system in question. By the methods just outlined, the constants  $k_1$  and  $k_2$  can be evaluated from the plot according to Equation 2 or Equation 4. From these, the corresponding Equations 1 or 3 can readily be solved to find the amount of antibody precipitated by any amount of antigen, even though the experimental points do not include amounts of antigen as large or as small as those substituted in the equation. In other words, the entire quantitative course of the serum, up to the equivalence zone or slightly beyond, can be predicted quite exactly from two or three experimental measurements in the region of antibody excess.

(2) The total antibody content of the serum, which is represented by the height of the curve B (Chart 3) is frequently not obtained directly, for the experimental points may have been made in the region of large antibody excess. If desired, the entire curve beyond the measured points may be calculated by assuming for Equation 1 or Equation 3 a series of antigen values and measuring the maximum height of the curve when plotted. The maximum may, however, be located much less laboriously by setting the first derivatives of the equations to zero, and solving. Thus, for Equation 1 we obtain

$$5 \quad k_1 - 2k_2 (\text{antigen}) = 0$$

where the desired value of antigen is that required to give the maximum of the curve. In particular, for the data of Table 14 this would be  $15.8 - 2(83) (\text{antigen}) = 0$ , the solution of which gives a value of 0.095. As with any other value for antigen, this can be

substituted into Equation 1 to obtain the corresponding (and maximum) amount of antibody. The answer, 0.752 mg. antibody N, is in excellent agreement (1 per cent) with the largest amount of antibody found in this experiment, 0.746. If Equation 3 is used in this instance, the first derivative becomes  $19.4 - \frac{3}{2} (36) (\text{antigen})^{1/2} = 0$ .

(3) It is often necessary to compare the quantitative reactivities of two or more sera of widely different antibody contents, for example, the first and later bleedings of an animal which has received extended courses of immunization. The existence of multiple combining proportions renders impossible the direct comparison at any one antigen level. However, by simple transformations (Heidelberger and Kendall, 1935) the data for all sera may be recalculated to some arbitrary total antibody content, usually 1 mg. antibody N per ml. This not only permits a direct comparison but often reveals unexpected regularities or irregularities which could never have been detected by relative titer methods (Treffers et al., 1942).

#### MECHANISM OF ANTIGEN-ANTIBODY REACTIONS

Consideration of the mechanism of antigen-antibody reactions has aroused much theoretical speculation and experimental ingenuity ever since the controversy at the beginning of this century over the rival views of Bordet, who favored the adsorption of antigen by antibody, and of Ehrlich, who stressed chemical reactions between the two. Although the views of the latter, considerably modified, find the greater favor today, offsprings of this argument still continue.

Equation 1 above is of particular interest in that it can be derived mathematically on the assumption that antigen and antibody combine in a series of bimolecular reactions which obey the law of mass action (summary in Heidelberger, 1939a, b; Kendall, 1942). A theoretical significance can be ascribed to the constants of the equation:  $k_1$  is numerically twice the value ( $2R$ ) of the ratio of antibody to antigen in the midpoint of the equivalence zone (designated R), and the constant  $k_2$  has the value  $R^2/A$ , where A designates the total antibody content of the serum. This interpretation makes it readily evident why the constant  $k_1$  is not dependent on the total antibody content of the serum, while constant  $k_2$ , which includes A, is.

The theory of Heidelberger and Kendall is a further development of a concept first proposed by Marrack (summary in Marrack, 1938), according to which each antibody molecule in a specific precipitate is surrounded by two or more antigen molecules to form a lattice type of aggregation. On this basis the so-called soluble, univalent or incomplete antibody does not precipitate after combining with antigen because it lacks the additional valence or valences necessary for aggregation into large, insoluble lattices. However, by means of this single valence it can attach itself to an insoluble lattice containing regular multivalent antibody.

It has been shown by Heidelberger and Kabat (1937) that the above equations also express quantitatively the reaction between antibody and bacteria in the agglutination reaction. In this, antibody acts as a bridge, connecting in specific chemical linkage antigen molecules contained on the surface of two or more organisms.

There is a considerable literature concerned with demonstrations of the specificity or nonspecificity of various stages of aggregation, and experiments have been devised to determine the valencies of antibody in specific precipitates. A number of other detailed quantitative theories have been proposed [Hershey, 1941; Pauling et al., 1943; Teorell, 1946; and Kendall, 1942 (antitoxin combinations)]. Some of the experimental data has been criticized by Boyd (1947) and Boyd and Behnke (1944).

#### THE DANYSZ PHENOMENON

The data of Chart 3 were obtained by adding to a constant amount of serum the



amounts of antigen indicated by the third column of Tables 13 and 14. Quite different amounts of precipitates are obtained if the antigen is not added to each tube in single portions but in successive small increments. The reason for this is of some interest, and at times, practical importance. Suppose that in tube No. 3 (Table 14) only one-fourth of the amount of antigen, or 0.020 mg., had been added initially; the proportions would then equal those in tube No. 1 and the same ratio of antibody:antigen precipitated, 14.2, would be expected. If the reaction were rapidly reversible the further addition of 0.020 milligram of antigen would result in a system equivalent to that in tube No. 2. Antigen-antibody reactions do not always reverse rapidly, however, and because of the variable combining proportions the system is then likely to act toward the second addition of antigen as if it contained relatively more antibody, i.e., a precipitate of higher antibody ratio will be formed. As further increments of antigen are added the process is repeated, the amounts of antibody precipitated continuing to be larger (up to a limit) than if the antigen had been added all in one portion. If sufficient time is allowed, however, the system will rearrange to the composition obtained by the one-step addition of antigen.

It can readily be seen that, if disproportionately more antibody is brought down by serial addition of antigen, less of the latter will be needed to reach equivalence. If the equivalence amount is determined in the usual manner but mistakenly added in successive portions to a second aliquot, an excess of antigen will result in the latter. This is of particular importance if the antigen is toxic. It is not necessary that antibody be inhomogeneous to behave as just outlined; it is sufficient that it be capable of combining in variable proportions and that the equilibria reached be not readily reversible. If the antibody is inhomogeneous, as it frequently is, an additional

effect is produced, for the serial addition of antigen may cause antibody of lesser reactivity to remain unprecipitated, even with excess antigen. The so-called soluble antibody (p. 185) can be demonstrated in this manner. Unless the soluble antibody is taken into account the total antibody content of the serum as determined by serial precipitations will be less than that determined by direct addition of antigen in one portion.

#### TOXIN-ANTITOXIN REACTIONS

In the serologic systems discussed up to this point the variable combining proportions of antibody with antigen have resulted in visible precipitation over a considerable range of antigen values. In the system described in Chart 3 and Tables 13 and 14 precipitation of antibody would have been evident with as little as 0.001 mg. antigen and was still not completely inhibited with 0.200 mg. antigen, a more than 200-fold variation. In qualitative tests this would encompass some eight tubes of twofold dilutions. This behavior is in sharp contrast to a number of serologic systems, of which certain toxin-antitoxin reactions are prominent examples. Thus if a precipitin titration is made with diphtheria toxin against its antitoxin produced in the horse, only one tube may show distinct flocculation while tubes containing one-half or twice this amount of antigen show little or no evidence of flocculation. Unlike the precipitin reaction discussed above, the toxin-antitoxin flocculation reaction exhibits inhibition with antibody excess as well as with antigen excess.

The *flocculation reaction* may also be carried out under the conditions necessary for quantitative absolute measurements. In Chart 4 are given the curves obtained when diphtheria toxin and scarlet fever toxin are employed as antigens against their respective antitoxins produced in the horse. In addition to the sharp zones of inhibition on either side, the curves have several proper-

ties which differentiate them from the precipitin reaction illustrated in Chart 3. The most important is that in the toxin-antitoxin reaction the entire amount of antitoxic antibody in the serum is precipitated at the beginning of the linear portion and the subsequent increase is due solely to the precipitation of antigen. The situation is therefore analogous to that in the equivalence zone of the precipitin reaction. This characteristic gives rise to two properties of definite practical usefulness.

(1) If all the antigen added precipitates, as it should if it is homogeneous, the antibody N can be obtained by subtracting the antigen N added from the total N found for any single point on the linear portion. There is thus no need to determine the curve either experimentally or by calculation from a few points as there was in the precipitin reaction. (2) Since the only additional precipitate on the linear portion of the curve comes from the antigen added, the amount of antigenically active material in a preparation of unknown purity may be determined by measuring the slope of this linear portion (i.e., finding the mg. N precipitated per mg. N added). For example, in the scarlet fever toxin system (B, Chart 4) the N precipitated by 500 flocculating units ( $L_f$ ) of toxin subtracted from that precipitated by 700  $L_f$ , when divided by the 200 toxin units added, gave a value of 0.00022 mg. N per  $L_f$  unit of toxin. Such assays may be made with toxins of any degree of purity (Pappenheimer and Robinson, 1937; Hottle and Pappenheimer, 1941).

The effect of serial addition of antigen on the composition of the resulting antigen-antibody precipitate has been described above. As was first noticed by Danysz, toxin-antitoxin systems also illustrate this point, even though no precipitate is formed with excess antigen or antibody. In such cases the serial addition of a quantity of toxin calculated from separate one-step experiments to be sufficient to neutralize a given quantity of antitoxin will result in a

mixture in which toxin is in excess, perhaps enough so as to cause death of an animal injected with it. If the mixture is permitted to stand for a few days it may rearrange to give a neutral mixture. The reaction has been postulated to go through a stage of soluble complexes which reverse slowly to more stable compositions (Pappenheimer and Robinson, 1937).

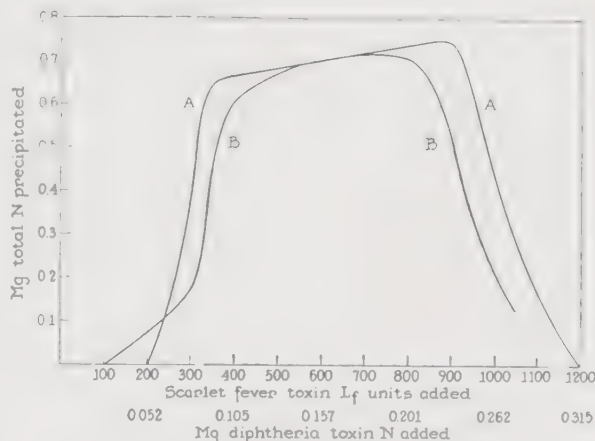


CHART 4. Quantitative toxin-antitoxin flocculation reactions. Curve A, diphtheria system. Curve B, scarlet fever toxin system. (Hottle, G. A., and Pappenheimer, A. M., Jr., 1941, A quantitative study of the scarlet fever toxin-antitoxin flocculation reaction. *J. Exp. Med.*, 74, 545-556.)

Much interest has been aroused by the inhibition zones with both excess antibody and antigen which are such a prominent feature of toxin-antitoxin flocculation reactions. Kendall (1942) has shown that if it is considered that antibody is bivalent and that the two dissociation constants for the antigen-antibody combination are not equal, values for the latter constants can be found which enable the reaction curves for typical examples to be calculated quite closely.

#### ANTITOXIN TITRATION

Before antitoxin can be employed in therapy its antibody content must be determined. Although antitoxic antibody may be assayed quite conveniently and accu-



rately in weight units from curves such as those of Chart 4, the standardization is more commonly done on the basis of arbitrary antitoxin units determined from animal protection experiments. To economize on the latter, provisional standardizations are first made with test tube flocculation tests or animal skin tests. The details of the official tests can be obtained in publications of the National Institute of Health or from standard manuals such as that of Wadsworth (1939).

Various units have been employed in standardizing toxin and antitoxins. For diphtheria toxin the minimal lethal dose (M.L.D.) is the amount of toxin which injected subcutaneously will kill a 250-gram guinea pig in four or five days. The  $L_0$  dose is the amount of toxin which will just neutralize one unit of an antitoxin which is kept as a reference standard by an official agency, such as the National Institute of Health. Due to the difficulties of determining when neutralization is just complete, this unit has been largely displaced by the  $L_+$  unit which is defined as the amount of toxin which, when mixed with one unit of antitoxin and injected subcutaneously, will kill a 250-gram guinea pig in four or five days. At first sight the  $L_+$  dose might be thought to be equivalent to the M.L.D. plus the  $L_0$  dose, but, as Ehrlich demonstrated, it is considerably greater. In fact, for some systems the difference between the  $L_+$  and the  $L_0$  dose may be 50 M.L.D. instead of only one M.L.D. (for specific illustrations see Zinsser et al., 1939, pp. 183-188). This follows from the multiple combining properties of antigen and antibody, and from the fact that toxin-antitoxin reactions are at least partially reversible, so that when the M.L.D. is added to the neutral  $L_0$  dose part of the newly added toxin is combined with antitoxin and less than a lethal dose remains free.

Other units, such as the serologic flocculation unit,  $L_f$ , and the intracutaneous skin test unit,  $L_r$ , are also employed in the pre-

liminary standardizations. If a toxin preparation does not contain toxoid, the  $L_f$  and  $L_+$  units should bear a constant relationship, generally near unity. The presence of toxoid will alter this and the potencies obtained from flocculation tests and skin tests will not agree. Antitoxin from certain horses may also show deviations in this ratio due to the presence of antibodies of more than one serologic reactivity.

## SEROLOGIC SPECIFICITY

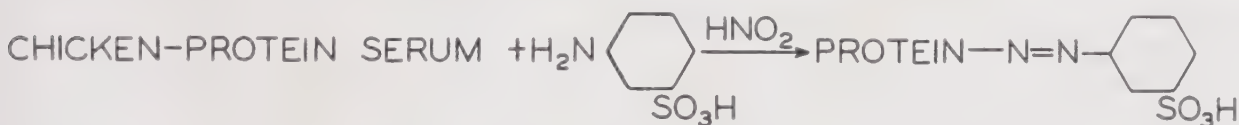
### CHEMICAL BASIS OF SPECIFICITY

Antigen-antibody reactions are characterized by a high degree of specificity. This specificity is due to chemical groups or units, and the degree of uniqueness of reaction given by an antigen depends on how widely distributed these particular constituent units are.

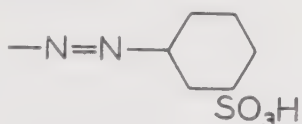
If the chemical structures of typical antigens such as bacterial proteins or polysaccharides were completely known it would be possible to account directly for differences and similarities in specificities. Our knowledge of the detailed structures of such materials is so incomplete, however, that very few conclusions of this type can be made. It is therefore necessary to employ more indirect approaches. The procedure usually adopted depends on the fact that although simple chemical compounds or radicals do not directly elicit antibodies on injection into animals they can modify or direct the antigenic specificity of true antigens to which they may be chemically attached. The researches influenced by Landsteiner over the past quarter century have systematically exploited this principle.

For a typical experiment we may select one designed to determine the influence on antigenic specificity of acid groups of different composition and spacial configuration. The "antigen" employed was chicken serum protein. Its sole function was to provide the basic high molecular weight unit

which would elicit antibodies. Its own complex specificities were unimportant, and, in fact, the experiment was so arranged that these would cancel out in the final readings. The acid groups to be studied were  $-\text{COOH}$ ,  $-\text{SO}_3\text{H}$ , and  $-\text{AsO}_3\text{H}_2$ , arranged in the ortho, meta and para positions about a benzene ring. These compounds are conveniently coupled to chicken serum antigen by means of the  $-\text{N}=\text{N}-$  diazo linkage. The coupling reaction may be written as



Other coupled antigens were prepared using the ortho and para isomers of aminobenzenesulfonic acid, and each was injected into rabbits. The specificity of the resulting antibodies could then be tested by the precipitin reaction. As test antigens, similarly coupled proteins were used with the exception that the protein portion was of a specificity other than that employed in the immunizing antigen, for example horse serum protein. Thus only antibodies to the common grouping



would be detected. The test antigens also included compounds in which  $-\text{COOH}$  or  $-\text{AsO}_3\text{H}_2$  replaced the  $-\text{SO}_3\text{H}$  group coupled to the immunizing antigen. In Table 16 are listed the reactions given by an antiserum to the sulfonic acid-protein complex. It should be noted that although the largest amount of precipitate was obtained with the homologous test antigen (containing the meta  $-\text{SO}_3\text{H}$  group) a significant cross reaction was obtained with the ortho isomer and to a lesser extent with the para isomer of this acid. When the acid group was varied, reactions could be obtained only with groups in the meta position, and of these the arsenic acid compound

gave a somewhat stronger reaction than the carboxylic acid.

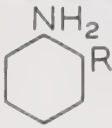
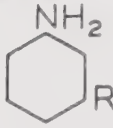
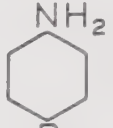
It has been possible with the aid of many ingenious coupling procedures to examine materials of different optical activities, such as *d* and *l* forms of tartaric acid, or  $\alpha$  and  $\beta$  glucosides, and to demonstrate that such configurations would determine largely or entirely the specificity of the antigen to which they were coupled. Studies with polypeptides coupled to proteins have shown

that the nature and positions of the amino acids in the chain exert a dominant influence. In all cases polar groups such as  $-\text{NH}_2$ ,  $-\text{OH}$ , and the various acid radicals were found to influence the specificity more than did the less highly charged aliphatic radicals. Landsteiner has termed the chemical unit coupled to protein, against which the antibody specificity is directed, a *hapten*. The term has since been broadened to include substances such as bacterial carbohydrates, which as isolated will react with antibodies to the entire organism but which cannot induce antibodies in all species, particularly the rabbit, although they may be antigenic in others, such as man.

It should be noted that the object of these studies was the determination of the influence of the groups selected on serologic specificity and that the reactions of other antibodies was minimized by the experimental methods employed. It is a matter of interest that in many of the animals injected relatively little of the antibody was directed solely against the specificity-modifying hapten; the reaction was rather with the entire combination of protein + hapten, a much larger serologic unit. An experiment in which this point has been considered in quantitative fashion has been reported by Haurowitz and Schwerin (1943). The literature on artificially conjugated antigens



TABLE 16. REACTIONS OF VARIOUS ANTIGENS WITH AN IMMUNE SERUM FOR META-AMINOBENZENE SULFONIC ACID (METANILIC ACID) \*

ANTIGENS CONTAINING			
	ORTHO	META	PARA
AMINOBENZENE SULFONIC ACID	+±	++±	±
AMINOBENZENE ARSENIC ACID	O	+	O
AMINOBENZOIC ACID	O	±	O

R designates the acid groups (COOH or SO<sub>3</sub>H or AsO<sub>3</sub>H<sub>2</sub>).

\* Landsteiner, K., 1945, *The Specificity of Serological Reactions*, ed. 2. Harvard University Press.

will be found in Landsteiner (1945) and in the extensive quantitative experiments of Pauling and his group (Pressman et al., 1946).

The differences in serologic activity which Landsteiner investigated were generally sufficiently great to be detectable by simple qualitative tests. There are nevertheless a number of interesting cases in which differences are not readily demonstrable unless more quantitative procedures are employed. As an example we may cite the antigenic activities of the  $\gamma$ -globulin from normal horse serum and the purified  $\gamma$ -globulin antibody from immune horse serum. If an antiserum is prepared in rabbits against either protein the endpoints obtained in precipitin titrations employing both antigens are so close together that the two appear to have the same antigenic specificity in spite of the fact that the molecular weight of the immune  $\gamma$ -globulin is six times that of the normal globulin. If the antibody removed by each is determined quantitatively it can readily be shown that these two  $\gamma$ -globulins from the same species have distinctly different antigenic properties (Treffers et al., 1942; Erickson and Neurath, 1945). As another example, Bendich et al. (1946) have shown that prepara-

tions of blood group A substance derived from different sources may differ quantitatively in their precipitating powers toward antisera, even though their gross chemical compositions are the same.

Most quantitative comparisons of antigens have been made in experiments in which an excess of antibody always remained. Instances are known, however, in which striking differences between antigens do not become evident until there is a considerable excess of antigen over antibody, i.e., mg. for mg. the two antigens have the same ability to precipitate antibody as far as the equivalence zone, or beyond, but they then differ in their abilities to bind the antibody in soluble complexes. Tobacco-mosaic virus and acuba mosaic virus form such a pair (Kleczkowski, 1941). Similar effects have been reported by MacPherson and Heidelberger (1945) for denatured egg albumins.

As Landsteiner was able to illustrate so lavishly with his experimental material, cross reactions may occur whenever two antigens have in common either identical determinant units or ones which are closely related. What constitutes a determinant serologic unit is not easy to define. There are indications that some units may be as

large as an entire molecule since in some sera a part of the antibody cannot be removed by any combination of lesser units; on the other hand, Landsteiner has obtained inhibition of precipitation with silk fibroin units of molecular weight 600, and inhibition reactions have been observed with simple chemical compounds of even lower molecular weight. Antibodies appear to be produced against a large number of serologic units, some of them overlapping, into which an antigen molecule may be divided.

The amount of cross reacting antibody may depend on the species of animal injected. This effect may be so large as to amount to a qualitative difference and markedly affect conclusions as to the similarities or dissimilarities of two antigens. For example, if horses are injected with the Type VIII pneumococcus (Pn VIII) the sera show a cross reaction with Pn III. The part cross reacting with Pn III may comprise 25 per cent of the total antibody; the remainder is strictly specific for the homologous Pn VIII. From this it would of course be concluded that Pn III and Pn VIII contain one or more specificity units in common. If however antisera to Pn VIII are produced in rabbits little or no cross reactions are obtained. The conclusion from this would be that the antigens were unrelated. Similar findings are obtained if antisera to Pn III are produced in both animals, and then tested with Pn VIII antigens.

Although other explanations are possible, we may consider that Pn VIII and Pn III contain a unit A in common (as is known from chemical evidence) and that horses respond to this serologic unit with the production of antibody which results in the observed cross reaction. If we suppose that unit A is not antigenically active in rabbits (it may resemble too closely a specificity unit which occurs naturally in rabbits) then the antibody which is produced in that animal after injection of Pn VIII will be to units such as B-G which are not possessed by Pn III, and similarly antibodies produced in the rabbit after injection of Pn III do not include antibodies to A, which is the antigenic unit possessed in common with Pn VIII. It is thus evident that antibodies to a chemically homogeneous antigen such as Pn VIII produced in the two species horse and rabbit may be qualitatively different in specificity since they are

produced to different serologic determinant groups.

An antigen I may exhibit a cross reaction with antisera to another antigen, II; however, even though the species immunized is kept constant, antisera to antigen I need not react at all with antigen II. Such puzzling nonreciprocal cross reactions may be accounted for if it is assumed that each antigen contains a unit A in common but that for some reason in antigen I the unit A is so situated that it cannot influence the antibody forming mechanism although it can still react with antibody formed by antigen II. Such relations have been observed with a number of pairs of antigens, such as the pneumococcus and the Friedländer B bacillus, between sheep blood and certain bacteria, and between synthetic conjugated antigens. Some evidence for at least the temporary masking of an antigen is provided for by the changes in combining proportions of antibody with continued immunization (p. 167).

The chemical building blocks from which protein and polysaccharide antigens are constructed, amino acids, sugar units, etc., are numerous but not unlimited in variety. Although it is not clear how many must be linked together to provide a natural determinant unit, the data do suggest that common serologic units are distributed among a great many antigens. How then can we say that an entire protein or polysaccharide molecule possesses a serologic specificity?—merely by defining the specificity as the unique sum of reactivities, part or all of which may be possessed, in different combinations, by other antigens. As an analogy, a house may be constructed of no material not found in neighboring houses and yet exhibit a distinctly individual character.

#### SPECIES AND ORIGIN SPECIFICITY

If a sufficiently complete serologic analysis is made, a given antigen derived from a particular species of animal can almost always be uniquely distinguished. This point finds application to many medico-legal problems, in which, for example, it may be of importance to distinguish human



from animal blood, or in the case of suspected food adulteration, beef from horse protein. Antigens are therefore said to possess species specificity. It should not be thought, however, that all antigens from a given species necessarily have common elements. Indeed there appears to be no "human" or "bovine" antigenic factor common to all human or bovine proteins, or probably to any pair of them. Even the albumin and globulin which occur together in blood serum are independent in serologic specificity and show no crossing whatever. In contrast, comparable proteins, such as the serum albumins from different species, often do show cross reactions the extent of which parallels to a large degree the closeness of the zoologic relationships, and this principle has been used to clarify systematic classifications in doubtful points. The extent of the crossing among antigens derived from particular organs such as the lens or the thyroid may be so large that the antigens are frequently said to possess organ rather than species specificity. Careful quantitative studies, such as those of Stokinger and Heidelberger (1937) on thyroglobulin have clearly demonstrated that in spite of this high degree of cross reaction the antigens nevertheless each possess individual characteristics. Absorption technics may be particularly valuable for establishing this point.

#### MIXED ANTIGENS

The antigens most conveniently studied are those composed of only a single molecular species. A number of crystalline proteins fit this criterion; the properties of one of them, crystalline egg albumin, have been described in Chart 3. Unfortunately, many of the substances of the greatest direct immunologic interest, such as bacteria and blood or serum, are not simple antigens but rather complex collections of them.

It can readily be seen that if a bacterium such as the streptococcus is injected into an

animal antibodies will be formed against each of the antigens present. It may be assumed as a working hypothesis that each antigen will react with its specific antibody according to curve A of Chart 3 [if horses were injected, some of the antibodies present might react to give a prozone (p. 170)]. If it could be assumed that the amounts of each antibody were exactly in proportion to the amount of that antigen injected, and if the combining ratios of all were identical, then the system would behave either by the quantitative absolute titration or by relative dilution titrations as a single antigen-antibody system. Except in very special instances, neither of these assumptions is true. Antigens present in mixtures do not produce equal amounts of antibody, nor are the relative amounts constant from individual to individual (p. 183). Antigen-antibody combinations also have characteristic combining proportions, both in the equivalence zone and in the regions of antibody and antigen excess. As a result, if mixtures such as bacterial culture filtrates or whole blood are tested as "antigens" against the antibodies produced to them the serologic reactions will be complex and difficult to interpret.

As an example, we may consider a mixed "antigen" composed of only two components, serum albumin and  $\gamma$ -globulin, present in the ratio of 3:1. Let us further suppose that the two are not equally antigenic but that for simplicity the same absolute amounts of antibody, 1.0 mg. N, are produced to each. If the combining ratio of antibody to albumin at the equivalence point is 5:1, then it will require  $1.0/5 = 0.2$  mg. of albumin to precipitate the 1.0 mg. of antibody N. A combining ratio of 8 for the  $\gamma$ -globulin will similarly require  $1.0/8 = 0.12$  mg. of this antigen for complete precipitation. If the antiserum is treated with small amounts, such as 0.10 mg. of the mixed antigen, the entire amount added of both antigens will be precipitated, while still leaving free antibody to each, as can be demonstrated by test on the supernatant with the single, isolated components.

Since the mixed antigen contains 75 per cent albumin, if a total of 0.26 mg. is added

to the serum in one portion, it will provide 0.20 mg. albumin or an amount just sufficient to precipitate all of the anti-albumin. The supernatant will still contain antibody to  $\gamma$ -globulin, however, since this amount of the mixture provided only 25 per cent  $\times$  0.26 = 0.066 mg. of  $\gamma$ -globulin, or little more than one-half of the equivalence amount for this component. If slightly larger amounts of the mixed antigen had been added in one portion there would be an excess of free albumin in the supernatant and positive tests would be given for free antigen (in reality, only for albumin) as well as for free antibody (to  $\gamma$ -globulin). If only the mixed components and the antiserum to them are available as reagents, it cannot be ascertained, of course, which of the components is giving the test for excess. Finally, if 0.48 mg. of mixed antigen is added to the serum, 0.12 mg. of  $\gamma$ -globulin will be provided, an amount just sufficient to precipitate completely the antibody to this second component. This amount of mixed antigen contains 0.36 milligrams of albumin, or an 80 per cent excess. How large a reduction in the amount of antialbumin precipitating will result depends on the combining proportions of this particular system.

Antibodies to bacterial polysaccharides generally are characterized by relatively flat reaction curves (greater than that in Chart 3) so that a twofold excess of antigen results in little reduction in the amount of antibody precipitated. Most antiprotein curves are well rounded, however, and show considerable inhibition under these conditions. The most marked effects are obtained with antibodies of the antitoxin type (p. 170) where a twofold excess of antigen will result in complete inhibition of precipitation. A simple dilution titration with a mixed system of this type may indeed show two or more distinct zones of flocculation.

In summary then, absolute antibody determinations on mixed systems have little quantitative meaning unless purified, single-test antigens are used since one antibody is very likely to be inhibited by the amount of mixed antigen necessary to bring down one or more of the other antibodies. Properly used, tests for antigen and antibody

remaining in the supernatant provide valuable information on the complexity of the antigens being tested.

## NATURAL ANTIGENS

Proteins and carbohydrates can act as antigens. Although lipids occur attached to antigenic material they do not appear to influence the specificity nor have purified lipids been found to elicit antibodies.

### PROTEIN ANTIGENS

Proteins are found everywhere in biologic material, not only as structural constituents but as enzymes, hormones, etc. Their serologic behaviors have been reviewed at length by Landsteiner (1945), Kabat (1943) and Treffers (1944). Only certain general properties need concern us here.

Blood is probably the most complex single "antigen" which has been employed in serologic studies. Well over 100 constituents have been identified, but not all are antigenically active.

Although the common heme portion is essential for the respiratory activities of hemoglobins, it apparently does not influence their antigenic specificities which are directed only toward the globin portions. As might be expected, more or less extensive cross-reactions are given by hemoglobins from related species. Blood plasma or serum contains a great many antigens. The material may be characterized chemically and physically in a variety of ways, of which the Tiselius electrophoretic analysis has been the most valuable (Tiselius, 1939-1940). Antigenic studies utilizing serum proteins as antigens have been difficult because of the number of components involved. The careful study of Kendall (1937) should be consulted for a rigorous approach to the problem.

**Bacterial Proteins.** Although many of the type-specific and group-specific antigens



of bacteria are polysaccharides, this is not invariably the case. Many important pathogens, including the streptococcus, the tubercle bacillus, and the cholera vibrio contain protein antigens and antibodies to some of these are protective. In addition, the typical bacterial exotoxins such as botulinus, tetanus, diphtheria (responsible for the Schick test), and the erythrogenic toxin of the hemolytic streptococcus (responsible for the Dick test) are proteins which have been isolated in purified form. Details on their chemical and serologic properties will be found in the reviews of Kabat (1943) and of Treffers (1944, 1947). In addition to toxins, bacteria also contain other protein antigens which frequently give rise to allergic reactions.

Nucleoproteins of both the ribose and the desoxyribose type have been isolated from most bacteria. Although antibodies to these are formed when either the entire organisms or the purified nucleoprotein fractions are injected into animals, the specificity appears to be directed only toward the protein portion, and the antigen-antibody reactions are not influenced by added nucleic acids (Stacey, 1947).

Extensive evidence has accumulated for the protein nature of *enzymes*. Almost all of the enzymes which have been examined have been found to be antigenic, and as is characteristic of antigen-antibody reactions the resulting antibodies have the property of precipitating the enzyme antigen from solution. Interestingly enough, this combination of antibody with enzyme need not completely neutralize the activity of the latter. Antitoxin to *Cl. welchii* will inhibit completely the lecithinase activity of *Cl. welchii*  $\alpha$  toxin, but antibodies to urease will only partially inhibit the activity of the latter enzyme. As extreme examples, antibodies to mushroom tyrosinase or to bacterial catalase do not affect at all the activities of these enzymes, which are retained in the specific precipitates. It would appear from this that the groups responsible for

enzymic activity and for antigenic (i.e., antibody-combining) activities are distinct. Analogous effects have been observed for some toxins from Gram-negative bacteria, and for antibody protein (in anti-antibody studies), in which one biologic property—toxicity, or the ability to combine with antigen—is more or less distinct from the ability to combine with antibody. Studies on antienzymes have demonstrated antigenic specificities for enzymes such as pepsins and trypsins derived from various sources (reviewed by Sevag, 1945). There is no doubt that animals injected with certain *protein hormones* derived from a second species may in time become refractory to such materials. There is some controversy, however, as to whether this is due exclusively to antibody formation. Nevertheless, definite instances of antibodies to protein hormones have been noted and, in the case of anti-thyroglobulin, carefully studied (Stokinger and Heidelberger, 1937). Fortunately for therapy the antigenicity of hormones appears to be low under the usual conditions of administration. The literature on this subject has been reviewed by Landsteiner (1945) and Chase (1945).

#### POLYSACCHARIDE ANTIGENS

These are composed largely of sugar units linked together into long threadlike molecules. The viscosity of their solutions provides some indication of the chain lengths. Glucose, mannose, galactose, glucuronic acid and glucosamine appear most frequently as constituents. Many polysaccharides contain acetyl groups, or phosphorus in some form. They are usually water soluble and non-dialyzable, and all are optically active. The molecular weights are not known with exactness (because of technical difficulties), but many are probably of the order of 100,000 or more. Soluble preparations certainly contain chains of varying lengths, and thus molecular sizes, which affect their serologic properties. The general properties of bac-

terial polysaccharides have been reviewed recently by Evans and Hibbert (1946) and those of mucopolysaccharides by Stacey (1946). Data on some representative polysaccharide antigens is given in Table 17.

In isolating bacterial polysaccharides, particularly those which contain acetyl groups, great care must be taken not to de-

given via the intact organism, it is evident that they have been modified, perhaps by rupture of a linkage to protein, by the isolation procedure. They do not give rise to pronounced systemic toxic effects in moderate doses.

Boivin has obtained a whole series of lipopolysaccharide antigens from Gram-nega-

TABLE 17. CHEMICAL PROPERTIES OF SOME REPRESENTATIVE SEROLOGICALLY ACTIVE POLYSACCHARIDES FROM BACTERIA

ORGANISM	$\alpha_D$	TOTAL N	P	ACETYL	SUGAR AFTER HYDROLYSIS, CALC. AS GLUCOSE
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Pneumococcus, Type I.....	+278	4.6	0	7.1	30
Pneumococcus, Type II.....	+55	0.14	0	0.4	80
Pneumococcus, Type XXII.....	+32	1.7	6.4	4.6	
S. dysenteriae (Flexner V).....	+22	6.1	1.5	6.0	40
S. aureus.....	+15	8.2	1.1		18
Friedländer bacillus, Type A.....	-103	0			64
Pneumococcus, group heterophile antigen...	+69	5.6	4.8	13.2	43

grade them, since this will affect their serologic properties. Although individual specific polysaccharides may constitute as much as 10 per cent or more of the weight of an organism, there is as yet no clear indication of what role they play in the cell's economy. Serologically active polysaccharides have been isolated from most pathogenic species, and from a great many nonpathogenic ones as well. As discussed elsewhere (p. 79), polysaccharides are frequently, although not invariably, involved in the pathogenicity of organisms and antibody to the polysaccharide may constitute the principal humoral defence against the organism.

The polysaccharides of certain bacteria, including the Gram-positive pneumococci and the Gram-negative Friedländer's bacilli, occur in capsules surrounding the organisms, but are readily released into the culture medium. As prepared in the laboratory they are usually antigenic in mice and in humans although not in rabbits. Since they are antigenic in the latter species when

tive organisms by extraction with trichloroacetic acid. They appear to be contained on the surface of the organism but do not form distinct capsules. They are antigenic in animals, although their toxicity necessitates the injection of rather small doses.

Antigens related to those of Boivin but distinct in composition have been obtained from many Gram-negative organisms by W. T. J. Morgan and his collaborators, who have used organic solvents such as diethylene glycol or phenol for the extractions. As isolated they are in the form of complexes containing a phospholipid, a protein, and the polysaccharide. They are of particular interest in illustrating the complexity of the antigen as it is attached to the cell, and of the necessity for having a protein carrier if antigenicity is to be secured in rabbits. When injected alone into an animal the protein part of the complex antigen gives rise to antibody which reacts specifically with itself. When the polysaccharide is coupled to it, however, most, if not all, of the



antibody produced now reacts only with this polysaccharide portion. This is further borne out by the lack of cross reaction between purified antigens from *E. typhosa* and *S. dysenteriae* which have common protein carriers but specific polysaccharides. The protein carrier has an additional practical usefulness in that many substances, such as gum or blood group polysaccharides or even gramicidin, may be converted into full antigens by conjugation with it (Partridge and Morgan, 1942; Morgan and Synge, 1945). Perlman and Goebel (1946) have shown for some members of the Flexner dysentery group that the cross reactions formerly thought to be due to two or more antigens contained in the organisms are actually due to multiple determinant groups on a single antigen molecule.

Although the administration of purified soluble polysaccharides, such as that of *E. typhosa*, has been reported to give rise to fewer toxic symptoms than are given by comparable immunizing doses of whole organisms, these antigens nevertheless possess a considerable toxicity. Unlike diphtheria or tetanus toxins or even the so-called soluble neurotoxin of *S. dysenteriae*, which are all proteins, these polysaccharide toxins cannot be detoxified with formaldehyde, but require more vigorous treatment with periodic acid (Goebel, 1947), or acetylation (Treffers, 1946).

Polysaccharides of this type have the further property that while their toxicity can be reduced somewhat by combination with antibody it cannot be completely abolished. As a result, if sufficient toxin—ordinarily a few multiples of the lethal dose in the absence of antibody—be used, no amount of antibody will suffice to protect the animals. This is in marked contrast to diphtheria and tetanus toxins, large doses of which can be completely neutralized by directly proportional amounts of antibody. The toxic polysaccharides of a wide variety of Gram-negative antigens also have the ability to cause hemorrhage in certain tumors.

The development of fever in humans after intravenous medication has been shown to be due in some instances to contamination of the solutions with minute amounts of Gram-negative micro-organisms. The essential *pyrogenic factors* have proved to be antigens of the type just described. Adequate testing of all biologics, including sera and vaccines intended for intravenous use, now includes measurement of their fever-producing properties in rabbits, which are very sensitive to these toxins. There is some doubt as to whether continued injection of pyrogens results in an acquired tolerance to these materials. Large scale tests with rabbits have not shown any such effect. However, clinical observations have indicated that humans after from 8 to 10 therapeutic bouts of fever induced by typhoid vaccine may require enormous doses before fever is again produced. The data may be reconciled by the observations of Beeson (1947) who has shown that if rabbits are injected with sufficient frequency, preferably daily, a marked tolerance can be noted. If the injections are discontinued the tolerance is soon lost. It does not appear to be related to antibody formation.

#### HETEROPHILE OR FORSSMAN ANTIGENS

Forssman first reported, in 1911, that injection of guinea-pig organs into rabbits gave rise to antibodies which lysed sheep erythrocytes. This phenomenon depends on cross-reactive portions of antigens which are found both in animals and in many micro-organisms. The term heterophile antigen now includes all antigens which show cross reactions when the species from which they are derived are too remote for such a reaction to be expected. A number of such systems are known. Some, such as that linking the *Macacus rhesus* monkey and the pig, or the horse and the rat are of little direct interest, while others such as that linking human blood cell antigens to antibodies produced against *Pneumococcus* type

XIV in the horse have definite medical applications since sera containing these antibodies may give rise to severe transfusion reactions (Finland and Curnan, 1938).

Heterophile antigens are found in certain human sera, both normally and in higher titer during the course of disease, particularly infectious mononucleosis (Paul-Bunnell test). An antibody with blocking activity, which prevents the agglutination of sheep cells by otherwise active antibody has been found in some cases of infectious mononucleosis (Levine and Gilmore, 1945). Heterophile antibodies may also be produced in humans after administration of horse or rabbit serum. The presence of agglutinins as well as lysins for sheep cells, and the failure of guinea pig kidney to remove the lysins differentiate the above from true Forssman antibodies.

At least some of the Forssman antigens are lipocarbohydrates. At certain stages of purification they are alcohol soluble, at others, water soluble. The heterophile antigen of the *Pneumococcus* is closely related chemically to the group-specific C substance, with the addition of bound lipid. The serologic differences are striking, however. As little as 2  $\mu\text{g.}$  of the heterophile antigen will inhibit the lysis of sheep cells by antibody, whereas 1,000 times this quantity of C substance is necessary for inhibition (Goebel et al., 1943). The biologic properties of heterophile antigens are reviewed by Buchbinder (1935) and by Landsteiner (1945).

## ANTIBODIES

### ANTIBODIES AS SERUM GLOBULINS

Antibodies are serum proteins which have been modified so as to react specifically with the corresponding antigens. The proof of this is based first, on their general similarity to the other serum proteins toward a variety of agents such as heat, alcohol and other reagents, and to proteolytic enzymes, and second, on the analysis of specific pre-

cipitates and of purified antibody solutions obtained from them (Marrack, 1938; Heidelberger, 1939a, b).

Unless the animal has been subjected to a prolonged course of immunization, the antibody does not constitute a significant portion of the total globulin. In unimmunized individuals, Heidelberger et al. (1946, 1947) found that rarely did the antibody level to pneumococcus Types I, II, or V exceed 1  $\mu\text{g.}$  of antibody N per 4 milliliters of serum analyzed, and was frequently sufficiently below to be undetectable. Antibodies to the group-specific C substance were often present in much larger amounts, 21 to 23  $\mu\text{g.}$  N per 4 ml. serum, which is probably due to the fact that this antibody would be formed in response to contact with any type of pneumococcus. After recovery from infection significantly larger amounts of antibody can be demonstrated. For 23 cases treated with sulfadiazine, Heidelberger and Anderson (1944) reported the following antibody levels per 4 ml. serum: antibody type-specific for the infecting organism, 1-498  $\mu\text{g.}$ ; antibody to pneumococcus C substance, 9-143  $\mu\text{g.}$ ; antibody to pneumococcus nucleoprotein, 3-10  $\mu\text{g.}$  The antibody level of normal isoagglutinins is also apparently quite low. In a small series of individuals studied by Kabat and Bezer (1945) the amounts of anti-A or anti-B which gave isoagglutinin titers of 1:16 or 1:32 ranged from 1 to 5.5  $\mu\text{g.}$  per ml. serum. After injection of A or B blood-group substances, titers as high as 1:512 were obtained, and the antibody content was found to have increased to 55 or 60  $\mu\text{g.}$  antibody N per ml. It was found, as an average of all tests, that the limiting amount of antibody required to give isoagglutination was from 0.1 to 0.2  $\mu\text{g.}$  antibody N per tube. With this data as a basis it is evident that in a serum with an isoagglutination titer of 1:1,000—which would be considered quite potent for this system—the absolute amount of antibody would be only 100 or 200  $\mu\text{g.}$ , or 0.1 or 0.2 mg. antibody N. Since normal



serum contains about 10 milligrams of protein N per ml., this particular antibody would represent only some 2 per cent of the total serum protein, which indicates why difficulty has been experienced in correlating antibody production with the chemical or physical measurements on whole serum.

Purified antibody may be prepared by taking advantage of the fact that less antibody is generally bound by antigen if the salt content of the medium is increased to 10 per cent. The antibody is first precipitated with its specific antigen in 0.9 per cent saline, the specific precipitate carefully washed with saline to free it of all other serum proteins, and then suspended in 10 per cent saline. From 10 to 20 per cent of the antibody may then be released into the supernatant. After removal of the precipitate containing the antigen bound to the residual antibody, the supernatant antibody solution may be dialyzed against physiological saline to rid it of excess salt. Its purity may be determined by assaying the percentage of N specifically precipitable. In favorable instances 100 per cent of the protein may be specifically precipitated by antigen (Heidelberger and Kabat, 1938). Modifications of the method include the use of bacterial organisms as antigen, or dilute alkali as the dissociating medium. Such solutions have been invaluable for the determination of the physical properties of antibodies.

#### ORIGIN OF ANTIBODIES

A large body of evidence indicates that the reticulo-endothelial system is involved in antibody production. Recently it has been shown that antibodies can be found in lymphocytes, although the experiments have been more conclusive with respect to antibody transport than to its manufacture (Ehrich and Harris, 1942, Dougherty et al., 1945). It has also been shown that adrenal cortical hormones may release normal

$\gamma$ -globulin as well as antibodies into the circulation through their effect on lymphocytes (Chase et al., 1946; White and Dougherty, 1945). Danish investigators, on the other hand, have provided evidence that the plasma cells are at least in part responsible for antibody manufacture (Bjørneboe et al., 1947).

The amounts of antibody which can be produced in response to minute amounts of antigen are quite large. Thus the total blood serum of an individual injected with two doses of 0.05 milligrams of pneumococcus Type II polysaccharide contained after 3 weeks 1,370 milligrams of antibody protein, or more than 25,000 times the weight of antigen injected (Heidelberger et al., 1946). This, as well as earlier evidence, leaves little room for the view that antigen becomes permanently incorporated into the antibody manufactured.

The role which antigen plays in the manufacture of antibody is not yet known, nor is it certain that the antigen must always be present at the time antibody is formed. According to the views of Brienl and Haurowitz, and of Mudd (summarized in Sevag, 1945) antibodies are synthesized using the antigen as a direct template. Pauling et al., (1940, 1942, 1943) have discussed some of the possible details of the process. In a radically different theory proposed by Burnet (1941) the antigen is assumed to effect the synthesis of a proteinase which in turn directs the synthesis of antibody. Whether or not the presence of antigen is necessary for continued synthesis of antibody it appears certain from isotopic tracer experiments that antibody protein is continually being turned over, as are the other serum proteins, at the rate of about one-half every two weeks (Schoenheimer et al., 1942; Heidelberger et al., 1942). Antibody continues to be formed even though the total level is declining, which implies that in this circumstance the rate of destruction exceeds the rate of formation.

These theories and experiments are of

particular interest for the important problem of whether the presence of antibodies years after apparent recovery from an infection always denotes the persistence of the antigen, perhaps in a living and potentially infectious state. There have been numerous instances in which infectious agents have been recovered under just such conditions, but the general question cannot be answered at present.

IMMUNIZATION PROCESS

Although many millions of individuals have been injected with antigenic material as prophylaxis against various diseases, our knowledge of the factors which influence the antibody response is still most incomplete. If sufficiently sensitive means are used, antibody may be detected as early as 24 hours after a single injection of antigen. Appreciable amounts do not appear in the circulation for several days, the maximum being reached at seven or even ten days. If a second injection is given, the antibody level drops (so-called negative phases of immunity) but rises rapidly again until the circulating antibody reaches a higher level than before. This cycle is repeated after each injection, although the maximum antibody titer may be reached more quickly. The antibody level which can ultimately be obtained may be quite high; in rabbits values approaching that of the normal total globulin content (10 mg. N per ml.) have been obtained, and when multiple antigens were used, the antibody level rose to as high as 18 mg. N per ml. serum (Bjørneboe, 1941). These are exceptional figures, but it is doubtful whether the possible limit is at all approached under the usual short courses of immunization. There is evidence that the body can simultaneously form antibody against many antigens. In one instance antibodies were detected to 32 out of 35 antigens injected.

There is an individual variation in response to combined vaccines which is worth

noting. Heidelberger et al. (1946, 1947) have studied the antibody levels of a number of males of military age, a group more uniform than the general population, after injection of soluble pneumococcus polysaccharides. The three polysaccharide types used were all contained in the same solutions so that the antigens were always given in the same relative proportions. Nevertheless, as is evident from Table 18, some in-

TABLE 18. RESPONSE OF HUMAN SUBJECTS TO INJECTIONS OF PNEUMOCOCCUS POLYSACCHARIDES \*

( $\mu$ g. antibody N per milliliter of serum, 5½ months after injection)

SUBJECT NUMBER	ANTIBODY TO		
	PN TYPE I	PN TYPE II	PN TYPE V
71	5	9.5	2.5
74	3	22	2.8
76	39	10.7	2.3
80	18	4.5	41.5
82	1.7	12.5	14.2
Average of 20	8.3	7.8	6.5

\* Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B., 1946, Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides. *Journal of Experimental Medicine*, 83, 303-320.

individuals responded better to one type than to another, others responded equally well to all three. The variations in absolute amounts of antibody produced also cover a considerable range. The average antibody level of the 20 subjects was about the same for each antigen, which indicates the difficulty of determining relative antigenicities unless a sufficiently large number of subjects is examined to rule out individual variations.

The route of injection may influence greatly the amount of antibody produced to a particular antigen. Thus, the intracutaneous or subcutaneous injection of pneumococci or streptococci into rabbits results mainly in antibody to the group-specific



nucleoprotein, while type-specific antibodies are produced after intravenous injection. The species injected also exerts an influence since the intracutaneous or subcutaneous routes are effective for the production of antibodies to polysaccharides in humans.

NORMAL ANTIBODIES

Antibodies may not infrequently be found in animals which have not been injected with the corresponding antigen. In humans, antibodies to pneumococcus C substance, to diphtheria toxin, and to many other antigens can be accounted for on the basis of subclinical infections, indeed the corresponding organisms can frequently be demonstrated in the nose or throat during the carrier state. In other instances no definite contact can be shown. For example, no satisfactory theory of the origin of the blood group isoantibodies has been formulated. As is mentioned elsewhere, these appear in the serum in a manner predictable from the

blood cell antigens, whose inheritance is known to be under genetic control. Hemagglutinins for many animal cells are also present in the serum of young chicks. The source of the corresponding antigens has not yet been ascertained, and a genetic origin of the antibodies cannot be eliminated at present.

PROPERTIES OF ANTIBODIES FROM VARIOUS SPECIES

With only one exception (hemolysins) all known antibodies produced in the rabbit have the same physical properties (Table 19). The serologic reactivity is also uniform in that all antigens which have been tested, including diphtheria toxin, give precipitin curves of the type of Chart 3, without the prozone in the region of antibody excess often observed with horse antisera (p. 170). Antibodies produced in the horse show a more complex behavior. The two types of reaction curves which have just been men-

TABLE 19. PHYSICAL PROPERTIES OF ANTIBODIES AND OF SOME NORMAL GLOBULINS IN THE SERA OF VARIOUS SPECIES

SPECIES	PROTEIN	ELECTROPHORETIC FRACTION	SEDIMENTATION CONSTANT	MOLECULAR WEIGHT *
			<i>Svedberg units</i>	
Cow	Pn antibody †	γ	18	910,000
Pig	Pn antibody †	γ	18	930,000
Rabbit	Normal globulin	γ	7	150,000
	Pn antibody †	γ	7	157,000
	Antibody to egg albumin			
	Sheep cell hemolysin		19	
Horse	Normal globulin	γ	{ Mainly 7 Some 18 }	165,000
	Pn antibody †	γ or β <sub>2</sub> = T	18	920,000
	Diphtheria antitoxin	β <sub>2</sub> = T	7.2	184,000
Human	Normal globulin	γ	7	156,000
	Pn antibody †	γ	7	195,000
	Isoagglutinins	β <sub>2</sub> = γ <sub>1</sub>	18	
	Wassermann antibody	β <sub>2</sub> = γ <sub>1</sub>	{ 7 19 }	

\* The molecular weight is obtained from a combination of the sedimentation constant and the diffusion constant, which may vary independently.  
† Pn antibody = antibody to the pneumococcus specific polysaccharide.

tioned correspond to distinctly different physical and chemical properties for the antibodies concerned (Table 19). The precipitin type of antibody is found typically after intravenous injection of whole bacteria, but recent evidence indicates that it may also be formed after intravenous injection of bacterial autolysates, or of certain soluble proteins such as rabbit serum globulin (Heidelberger, 1947; Treffers et al., 1947a, b).

It has been the experience of laboratories producing diphtheria and other antitoxins that the flocculating type of antibody (p. 170) is produced only after the subcutaneous injection of the antigen. This variety of antibody may also be formed after the subcutaneous injection of proteins such as egg albumin or rabbit serum albumin. In contrast, when rabbit serum globulin is injected as an antigen by this route it gives rise solely to a third form of antibody—of the so-called soluble, incomplete or univalent type. Such antibody may also be formed during the early stages of immunization to other antigens. Thus serum taken from a horse which has received subcutaneous injections of crystalline egg albumin will not flocculate with any proportion of antigen. If, however, this serum is added to a flocculating serum of the same specificity (obtained at a later stage of immunization, or in some cases by altering the route of injection) a larger amount of precipitate is formed on addition of antigen:

Serum I (nonflocculating) + .10 mg. antigen N =	0 ppt.
Serum II (flocculating) + .10 mg. antigen N =	.60 mg. ppt. N
Sera I + II mixed together + .10 mg. antigen N =	1.40 mg. ppt. N
∴ Serum I contains, by difference, at least	.80 mg. antibody N

This form of antibody occurs in rabbit sera as well but apparently always with enough complete antibody so that a precipitate is formed on addition of the antigen. If the

latter is added in small increments, however, the usual form of antibody will be completely removed, together with a part of the incomplete antibody, and the supernatant can frequently be shown to behave like Serum I above. The biologic significance of this form of antibody is not clear and no information is available as to its protective function. Interest has been aroused recently in its possible connection with Rh blocking antibody in humans (p. 194).

When human serum is fractionated by the methods of Cohn (Edsall, 1947) antibodies to mumps and influenza viruses, staphylococcal toxin and the H antigen of *E. typhosa* are concentrated in Fraction II, consisting largely of  $\gamma$ -globulin, while the antibody to the typhoid O antigen and all of the isoagglutinins are concentrated in Fraction III-1, which contains 70 per cent of  $\beta$ -globulin and 25 per cent of  $\gamma$ -globulin (Enders, 1944). This is the first demonstration, based on chemical properties, of differences among human antibodies. More recent investigations (Deutsch et al., 1947) have shown that the typhoid O antibody and the isoagglutinins migrate in a separate electrophoretic fraction, which they termed  $\gamma_1$ -globulin. It appears to correspond to the  $\beta_2$  or T component of horse serum. The isoagglutinins in the  $\gamma_1$ -globulin are further distinguished by the fact that they are heavier and sediment with a velocity of 18 s, although quantitative absorption experiments indicate that in human serum only a portion of this heavy material is composed of isoagglutinins. At least a part of the antibody responsible for positive Wassermann reactions in the serodiagnosis of syphilis has the same sedimentation constant and electrophoretic mobility as the isoagglutinins, while the remainder has the same sedimentation constant, 7 s, as does the bulk of the serum globulin (Davis et al., 1945).

Preparations of Fraction II represent a 20-fold concentration of the  $\gamma$ -globulin over



that in plasma. Assays by relative titer methods have shown that the antibody levels of neutralizing antibodies toward influenza A virus approximate those obtained in convalescent sera; the titers of complement-fixing antibodies to mumps virus are somewhat less than those found after convalescence (Enders, 1944). As near as could be ascertained from the limited amount of data on convalescent sera, the antibacterial antibodies in the  $\gamma$ -globulin concentrate also approximated those found in the serum after recovery from disease. Numerous studies are appearing which indicate that  $\gamma$ -globulin concentrates have definite therapeutic value in the prevention or treatment of measles and other infections.

Mention should be made of an observation of Tillett and Francis who found that irrespective of the etiological agent, sera from the acute stages of many infectious diseases such as rheumatic fever, pneumonia, osteomyelitis and subacute bacterial endocarditis react with pneumococcus group-specific C substance. The reactive material disappears with the onset of convalescence. Further study showed that calcium was specifically needed for the formation of a precipitate and that the material occurred in the albumin rather than the globulin fraction of the serum. All of these points differentiate it from an antibody of the usual type. The protein responsible for the reactions has been isolated in crystalline form by McCarty (1947).

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## 8

# Blood Groups

### HISTORY

The possibilities of transfusing human or animal blood into humans were investigated as early as the eighteenth century, and several successful instances are recorded. However, some individuals developed severe shock on the first attempt at transfusion, and many of these died. Others, apparently luckier, survived one or more of the crude transfusions only to fall victim on later attempts. In consequence of these fatalities transfusions were abandoned as a general therapeutic procedure until the beginning of this century when adequate criteria were established for selecting donors.

The reactions which attended transfusions of animal blood into humans were the first to be accounted for, through the researches of Nuttall and others from 1880 to 1900. It was found that the blood of each species was characterized by individual antigens in both the cells and the serum. Moreover, even animals of the same species differed in the antigenic constituents of their erythrocytes (reviewed in Landsteiner, 1945, and Irwin, 1946). When humans were injected with blood from a foreign species reactions were not generally evident at first, although some individuals subsequently developed serum sickness (p. 130). If, however, an interval of ten days or more, sufficient for the development of antibodies, elapsed and a second trans-

fusion was then attempted, reactions were immediate and frequently severe or fatal. Sensitization persisted for months or even years.

The more interesting case—the reactions after transfusion with human blood—was shown to be due to the same cause through the classic demonstration by Landsteiner and his students that human bloods could also be divided into distinct antigenic types, of which four were found, and that antibodies (isoagglutinins) to these antigens were widely distributed in human sera.

### TRANSFUSIONS

Whole blood may either be administered directly from one individual to another, or, as is more frequently done in modern practice, it may be taken as needed from blood banks in which the sterile, citrated blood can be stored in the cold for periods of as long as a month. Only the details pertaining to the serologic aspects of this procedure will concern us here.

In transfusions of whole blood, or its components, cells or plasma, care must be taken that the donor's cells are not agglutinated by any antibody present in the recipient's serum, which is in large excess, and that the recipient's cells are not agglutinated by the serum incidently introduced with the donor's cells. Such agglutination, together with hemolysis of the cells in the



presence of serum complement, leads to blocking of capillaries and the release of hemoglobin into the circulation, as evidenced by chills, hemoglobinuria and other symptoms. If the plasma for transfusion is derived from pools of blood of various types, there is usually adequate absorption of the isoagglutinins by the heterologous types.

### BLOOD GROUPING AND CROSS-MATCHING

Blood is grouped by determining which of the A-B, the Rh, and for medicolegal work, M-N antigens are present in the cells by means of the agglutination reaction with potent sera of known specificity. The cells are chosen by preference since the antigens are present even at birth and remain identifiable throughout life. As will be evident shortly, the A-B grouping of any blood can also be determined by testing the serum for the agglutinating antibody or antibodies contained therein. Although this is a valuable check on the group as determined from the cells, it is not generally conceded to be reliable as a sole method of grouping since the content of antibody in the serum may be low, and the reactions difficult to demonstrate. This is particularly true for all groups in infants, and for certain of the subgroups such as A<sub>2</sub>B in adults.

After it is determined that both donor and recipient are of compatible groups a further check is made by mixing together the donor's cells with the recipient's serum, and also the donor's serum and the recipient's cells. No agglutination should occur in either case. In addition to safeguarding against serologic or clerical errors in grouping, this cross-matching may occasionally reveal incompatibilities not due to the usual blood groups. The technic of grouping and cross-matching is simple and rapid, but the details and precautions are of great importance. Thus particular care must be taken to exclude pseudoagglutination or rouleaux formation due to the use of too

concentrated sera, and nonspecific agglutinations due to autoagglutinins which appear in certain diseases such as paroxysmal hemoglobinuria, syphilitic cirrhosis, trypanosomiasis, etc., as well as panagglutinins present in normal human sera which agglutinate human cells of all groups when these have been exposed to the action of certain bacteria. Details of the usual practice will be found in standard works, such as those by Wiener (1943) or Schiff and Boyd (1942).

### A-B BLOOD GROUPS

This series, first described by Landsteiner and his associates in 1900 and 1901, is the only one with inherited isoagglutinins. In most instances it is still the only one routinely determined, although the others, particularly the Rh series, are being determined with increasing frequency. There are two antigens, A and B.\* The cells may contain either antigen alone, both, or neither, which gives rise to the groups A, B, AB, or O. The properties of these groups, summarized in Table 20, may easily be remembered from the following: (1) the group designation is that of the antigen or antigens contained in the cells; (2) an individual of any group will contain in his serum antibodies to those antigens *not* present in his cells; (3) an antigen added to the corresponding antibody (i.e., A with anti-A) forms an incompatible system.

Individuals of the same group are obviously compatible since the serum of any individual will not contain antibodies to his own cells (except as noted above) or to those of other members of his group. Individuals of group O are said to be universal donors since their cells cannot be aggluti-

\* This is not quite exact, although it is usually expressed in this form and is simpler for the purpose of remembering the reactions. Recent investigation has shown that, as long suspected, there is a definite antigen in group-O cells and that antibodies to it may be found in cattle sera, or in some human sera of groups A<sub>1</sub> or A<sub>1</sub>B (Witebsky and Klendshoj, 1941; Morgan and Waddell, 1945).

TABLE 20. PROPERTIES OF CELLS AND OF SERUM OF VARIOUS HUMAN BLOOD GROUPS  
INTERNATIONAL SYSTEM OF NOMEN-  
CLATURE \*

GROUP DESIG- NATION	RED CELLS		SERUM	
	ANTIGEN CON- TAINED	REACT WITH SERA OF GROUP	ANTIBODY CON- TAINED	REACTS WITH CELLS OF GROUP
A	A	B, O	Anti-B †	B, AB
B	B	A, O	Anti-A ‡	A, AB
AB	A, B	A, B, O	None	None
O	None §	None §	Anti-A Anti-B	A, B, AB

\* Other systems have also been used in the past. In the Moss system, International group A becomes Type II; B, Type III; AB, Type I; and O, Type IV. In the Jansky system the types are II, III, IV and I, respectively. The International system is to be preferred since it gives at once the antigenic composition of the cells, from which the reactivities can be determined as explained in the text.

† Frequently designated as  $\beta$  antibody.

‡ Frequently designated as  $\alpha$  antibody.

§ See footnote on page 190.

nated by the anti-A or anti-B antibodies present in the serum of individuals of groups B and A respectively. However, the serum of group-O individuals carries such anti-A and anti-B antibodies, but unless the donor's blood is given in large amounts the final concentration of these antibodies, diluted out in the recipient's blood, is usually too low to result in agglutination. A transfusion of 500 ml. of O blood into a 150-pound individual of group A will result in a dilution of the transfused antibody of about 1:10. If the O blood contains an anti-A titer greater than this (i.e., will agglutinate at higher dilutions) then it should be given with extra caution, even though some reports (Aubert et al., 1942) indicate that considerably higher titers have been given without untoward effects, presumably due to the neutralization of the isoagglutinins by soluble blood-group sub-

stances present in the plasma of the recipient. If "universal donors" are used in emergencies when other donors are not available, an estimate of the titer should always be made and compared with the final dilution obtaining after transfusion. Individuals of group AB are considered "universal recipients" since their sera do not contain antibody capable of agglutinating donor cells of any type. If other than AB cells are to be given to an AB recipient, the possibility of a high titer of anti-A or anti-B antibodies in the donor's serum should be considered.

The specificity A can be subdivided into two or more serologic subtypes. Thus, serum of group B (containing anti-A) may be absorbed with  $A_2$  cells until it no longer reacts with them, but still possess reactivity for  $A_1$  cells. Either  $A_1$  or  $A_2$  may occur together with B, to give the Groups  $A_1B$  or  $A_2B$ . The  $A_2$  reactivity may be difficult to detect, particularly when it is in combination with B. Further subdivisions into  $A_3$  and  $A_4$  have been made but these appear to be very rare.

## BLOOD GROUP SUBSTANCES

Although the blood-group antigens are present in the erythrocytes and can be extracted with lipid solvents, they are more conveniently prepared for study from other sources such as peptone, hog-gastric mucin or horse saliva which contain certain chemically related materials of this specificity. Chemical analyses indicate that the substances of A, B and O specificity are also very closely related chemically. They are polysaccharides containing an amino sugar, and amino acid components. The latter appear to be essential for antigenic activity. The blood-group substances are extremely stable, and blood dried for many years can be grouped by virtue of its content of these antigens. It has even been possible to group mummies of very ancient origin.

The blood-group substances are found



widely distributed in various organs of individuals of groups A and B. The occurrence in the saliva appears to be a special case controlled by a pair of Mendelian genes, and the individuals in such instances are said to be "secretors." The isolated, soluble blood-group substances have found some clinical application in the neutralization of isoagglutinins, so that emergency transfusions can be made of serum or plasma which would otherwise be incompatible (Witebsky et al., 1941). Significant amounts of these substances are also contained in plasma so that when plasmas of various groups are pooled in the proper proportions all of the antibodies may be neutralized by the soluble substances and thus rendered ineffective for isoagglutination.

### ISOAGGLUTININS A AND B

Isoagglutinins are not generally evident at birth but appear in increasing titer during the first few months. The titer varies during the life of an individual but the specificity of the antibody is always constant and consistent with the group as determined from the cells. As has been mentioned previously (p. 181), the actual quantity of isoagglutinin normally present amounts only to a few micrograms of antibody protein. They appear in a chemical subgroup (Fraction III-1 of Cohn) distinct from most of the other  $\gamma$ -globulin antibodies.

For routine blood-grouping tests, where active anti-A and anti-B antibodies are necessary, sera from individuals of groups B and A should be selected on the basis of a high titer of antibodies. For the preparation of typing sera of the highest activity, the titer may be increased above the normal by the injection of human volunteers of the proper groups with dried plasma containing the soluble blood-group antigens (Aubert et al., 1942) or with the purified soluble antigens (Witebsky et al., 1944). As

Wiener (1945) has pointed out, this immunizing effect of plasma may be quite undesirable in some cases since the resulting high titer of anti-A or anti-B antibodies may lead to conditions analogous to *erythroblastosis fetalis* due to the Rh factor, and this point should be kept in mind in giving plasma transfusions to women of child-bearing age, or younger. If animals are employed for the preparation of typing sera, it is necessary to absorb the sera with bloods of other groups since all human cells contain common "human" antigens which would otherwise give rise to non-group-specific agglutinations.

### INHERITANCE OF BLOOD GROUPS

The inheritance of the blood groups follows Mendelian laws. Each parent contributes one gene, which, in the case of the A-B series, may be either A, B or O (recessive). If a child receives the factor A from each parent his genotype becomes AA, and he will belong to blood group A. If the factor A is received from one parent and the factor O from the other, the dominant A prevails to determine the group as A. The genotype AO is heterozygous and unlike the previous case either the dominant A or the recessive O may be passed to the offspring. Similar considerations apply to the pair of genes B and O. Combination of the factor A from one parent with B from the other results in offspring of group AB. Group O can only result if both parents contribute this recessive factor.

Tables have been prepared (Boyd, 1947) which list the blood groups of offspring possible from mothers and fathers of given groups. For the A-B series there are 21 different combinations. In some, such as those between groups A and B, the offspring may belong to groups A, B, AB or O, depending on whether each parent is homozygous or heterozygous. This type of data is of great value in certain medicolegal problems, such as those involving disputed parentage or

interchange of infants. It must be emphasized that evidence from blood groups is of significance only when it is shown that a given offspring is incompatible with an alleged mating, and is generally legally admissible only under this condition. If the A, B and O antigens alone are examined, the chances of excluding any individual are somewhat limited, although this depends to some extent on his group. For example, if a putative father is of group A, then on the average an exclusion may be made only once in 17 instances. The probability of application may be greatly increased if the M-N group, the subgroups A<sub>1</sub> and A<sub>2</sub>, and particularly the numerous Rh subtypes are taken into account. All these form independent hereditary systems. By their means, over 100 serologic types can be distinguished for human bloods.

The percentage of individuals belonging to the different groups in the A-B, M-N and Rh series varies with different races. Thus, for the first series the distribution in the United States is about 41 per cent A, 10 per cent B, 4 per cent AB and 45 per cent O. Among Oriental races group B may be twice as frequent as group A, to give a distribution (in Hindus) of 19 per cent A, 41 per cent B, 9 per cent AB and 31 per cent O. Many tribes of full-blooded American Indians, on the other hand, are almost exclusively of group O. Similarly, the frequency of distribution of the Rh antigen in a North American population is about 87 per cent, but in some races, such as the Chinese, it exceeds 99 per cent. Data of this type are of great interest to anthropologists investigating the homogeneity of isolated tribes, but the correlation is a statistical one and no prediction as to an individual's race can be made from his blood group. To borrow an analogy: "Yale men as a group are taller than the national average, but it does not necessarily follow that the first tall man you meet did, or the first short man did not, go to school in New Haven."

### M-N ANTIGENS

In 1927 Landsteiner and Levine discovered two additional antigens, which they

designated M and N. All human erythrocytes contain one or the other factor, or both, which gives rise to the genotypes MM, NN or MN. There is, thus, no group corresponding to the recessive group O. With the very rare exception of anti-M, no agglutinin to these antigens is found in human sera, and this series does not constitute a problem for transfusion incompatibilities. Nevertheless, since at least one of the M-N factors is present in all individuals and they are inherited according to the usual genetic laws, they constitute an additional "tag" for characterizing individual bloods. The grouping technic is similar to that for the A-B antigens except that properly absorbed sera prepared in animals must be used.

### R<sub>H</sub> ANTIGENS

Although the existence of a factor common to human and rhesus monkey cells was discovered by Landsteiner and Wiener only in 1940, a voluminous literature has already grown on the subject, due to its medical applications. The nomenclature of the antigens is confusing and not yet standardized. At least six genes are now distinguished. Each chromosome contains one or two of the so-called Rh genes C, D or E, and one or two of the so-called Hr genes c, d or e, to make a total of three per chromosome. The Rh<sub>0</sub> type of Wiener may thus be written as cDe, the Rh' type as Cde, and the Rh'' type as cdE. Combined types which act as unit genetic characters are Rh<sub>1</sub> or CDe, and Rh<sub>2</sub> or cDE. The recessive Rh negative genotype is written as rh or cde (i.e., positive only for Hr). An individual who is homozygous for the Rh factor will contain a pair of identical chromosomes of one of the genotypes just listed; a heterozygous individual will contain a pair of dissimilar chromosomes, either (but not both) of which may be transmitted to a given offspring. As has been mentioned, about 85 per cent of the white European



and American population contains one or more of the Rh factors represented by the capital letters. Antisera are known to each of the six antigens whose presence is governed by the genetic factors. Excellent introductory surveys have been given by Diamond (1945) and by Boyd (1945) and in the more specialized accounts of Wiener (1946), Levine (1945) and Fisher (1947).

As with the M-N group, no antibody to the Rh factor is normally found in human serum. However, antibodies to any of the Rh antigens can be produced in man. Thus, an Rh negative individual (with no antigens of this group except the Hr factors in his cells) may receive a transfusion of otherwise compatible cells containing the Rh factor (Rh positive cells). If the individual has had no previous contact with this antigen, there will be no corresponding antibody present, and no untoward reaction. Antibody will now be formed, however, and if at a later time a second transfusion of Rh positive cells is given, the individual may exhibit the typical signs of transfusion incompatibility. A large percentage of the hitherto unexplained transfusion reactions in cases with proper A-B matching can now be accounted for on this basis.

An even more dramatic development has been the finding that an Rh-negative mother may produce antibodies to Rh antigens which leave the fetal circulation and pass through the placenta in some, although most fortunately not all instances in which the child is Rh positive (by inheritance from its father). These antibodies may in turn traverse the placenta and the child may be born with congenital hemolytic anemia, or *erythroblastosis fetalis*, or it may be stillborn (Wiener and Sonn, 1946).

## BLOCKING ANTIBODIES

Race (1944) and Wiener (1944) demonstrated independently that Rh-positive cells, after being exposed to certain human sera which were expected to contain anti-Rh antibody, were not agglutinated although they were rendered refractory to subsequent agglutination by sera with fully active antibody. This indicated that the first sera contained an antibody which, although not capable of causing agglutination, could add to cells in such a way that further combination with the usual antibody was blocked. The name "blocking antibody" was therefore given to it. Shortly thereafter, Coombs et al. (1945) provided additional evidence that an antibody had indeed added to the cells by the demonstration that such "sensitized" although unagglutinated cells could be agglutinated with a rabbit antiserum to human globulin. This test also proved to be a more sensitive means of detecting the "blocking antibody" than the preceding one.

In the usual test for anti-Rh agglutinins in human sera the test cells are added as a 2 per cent suspension. It has been shown, however, that if the Rh positive cells are added undiluted as whole blood, or if the cells are diluted with a protein such as plasma or serum albumin, even "blocking antibody" will cause active agglutination (Cameron and Diamond, 1945). In some individuals the "blocking antibody" may be the only one demonstrable, at least during part of the isoimmunization, or in others it may occur simultaneously with complete agglutinating antibody. By analogy with other systems (p. 185), the "blocking antibody" has been described as soluble or "univalent" in contrast to "multivalent" agglutinating antibody. Clinically, it appears to be as damaging as is the other.

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## 9

# The Diphtheria Bacilli and the Diphtheroids

### CORYNEBACTERIUM DIPHTHERIAE

#### INTRODUCTION

Wilson and Miles (1946a) define this group of micro-organisms as follows:

Gram positive rod-like forms, arranged usually in a palisade. Not acid fast. Often with club shaped swellings at the poles, generally with irregularly staining segments or granules. Nonmotile, nonsporing. Growing aerobically or under microaerophilic conditions, but often capable of anaerobic cultivation. Never forming gas in carbohydrate media, in which they may or may not produce acidity. They may or may not liquefy gelatin or serum. Some species produce a powerful exotoxin. Type species, *Corynebacterium diphtheriae* (Figs. 1D and 2D).

Although representatives of this species are widely distributed in nature, the majority appear to be associated with the body surfaces and tissues of animals and man. A few are of possible significance in veterinary pathology, but in human disease the group of closely related forms known collectively as the diphtheria bacilli (*C. diphtheriae*) occupy a position of outstanding and unique importance.

In many respects diphtheria represents the disease in which the bacteriologist may feel the pride of maximal achievement. Most of the facts regarding it—etiology, mode

of transmission, mechanism of pathogenesis, therapy and prevention—have been thoroughly elucidated. Difficulties in application of our knowledge, as well as some remaining lacunae in the facts themselves, nevertheless permit the disease to continue and occasionally to assume formidable proportions.

To a considerable degree, the success realized in the understanding and control of diphtheria results from the circumstance that it is a purely toxic disorder, uncomplicated, at least in general, by invasion of tissues by the micro-organism. It may be considered as providing the pattern for the understanding of a group of diseases such as tetanus and botulism, which closely resemble it in mechanism of pathogenesis, and of a further group including scarlet fever and gas gangrene, in which specific toxins are responsible for an important element of the disease process. For these reasons, it seems appropriate to select diphtheria as the first of the infectious diseases to receive detailed consideration.

#### HISTORY

The occurrence of diphtheria in epidemic form dates far into antiquity (Holmes, 1940). It was undoubtedly confused at times with other pathologic conditions af-

fecting the throat, mouth and adjacent tissues; streptococcal or fungal infections, Vincent's angina, nutritional disturbances such as scurvy, and others, alone or superimposed upon diphtheritic infection, must have presented extraordinarily perplexing problems in diagnosis to the early physician. In spite of these difficulties, certain symptoms uniquely characteristic of diphtheria, especially the paralysis of the soft palate with resulting regurgitation of fluid through the nose in attempting to swallow, indicate clearly that the disease existed in the sixth century and had probably even then been known for hundreds of years. It was not until the early nineteenth century that the French physician Brettoneau placed the specific clinical diagnosis of diphtheria on a reasonably firm foundation, which, although surprisingly accurate, could not become entirely so until supplemented by the discovery of the diphtheria bacillus by Klebs in 1883 and the demonstration of its etiologic relationship to the disease in 1884 by Loeffler. Frequently referred to in honor of these early bacteriologists as the *Klebs-Loeffler bacillus*, or colloquially, "K. L.," this organism, or group of organisms, has thus been recognized in its relation to infectious disease since the first decade of medical bacteriology.

In 1888, Roux and Yersin demonstrated that the diphtheria bacillus exerts its disease-producing effect by the formation of a soluble poison, an exotoxin, which is produced in culture under suitable conditions, can be separated from the bacterial cells themselves, and is capable of producing in animals the symptoms and type of death characteristic of infection with the organism. This clarified the mechanism of pathogenesis in man and explained the occurrence of the neurologic symptoms, the cardiac failure and other manifestations of the disease remote from the local and more obvious process in the throat. Within a short time, Behring found that the animal body was capable of responding to minute

sublethal doses of this toxin by the elaboration of a substance capable of specifically neutralizing the poison, an antitoxin, and in 1891 sera from immunized animals were first employed in the treatment of clinical diphtheria. The foundation was thus laid for a half century of specific serum therapy and prophylaxis not only of diphtheria, but of a variety of other infectious diseases.

The methods for controlling diphtheria in populations by mass immunization developed logically from these early observations. Suggested by Theobald Smith in 1909, toxin neutralized by antitoxin was shown by Behring in 1913 to induce immunity safely in both animals and man, and was applied on a large scale by Park (1922) for the protection of children. A simple test for immunity by the intracutaneous injection of minute amounts of toxin was developed in 1913 by Schick, making it possible to define more accurately the need for and the results of artificial immunization. Finally Ramon, in 1923, showed that formalin-treated toxin, *anatoxin* (now commonly called *toxoid*), possessed certain advantages as an immunizing agent over toxin-antitoxin mixtures, and this material, in one form or another has been used for wholesale immunization, especially of children, in many communities. Thus the knowledge and tools appear to be at hand for eradicating diphtheria. A great deal has been accomplished, but aside from the expense involved, and the very magnitude of the undertaking, there are still unsolved difficulties and unknown facts which may thwart even the most active public health campaign.

#### MORPHOLOGY

The characteristic feature of the corynebacteria, from which they derive their name, is their varying diameter, often broader at one end than the other, resulting in a "club" shape. A further characteristic depends on an irregular distribution of protoplasm within the cell, causing un-



even absorption of certain dyes which result, when suitably stained, in a beaded or barred appearance of the organism. This may be particularly noticeable in the case of certain strains of the diphtheria bacilli, often accentuated by growth on special media and leading to the appearance of well-defined "polar bodies." These deeply staining bands and beads have been variously named metachromatic granules, Babes-Ernst bodies, etc. Their true significance and their chemical nature remain unknown. They have been thought variously to consist of nucleic acid or of food reserves. Corynebacteria are Gram-positive, non-spore-bearing rods without flagella or capsules, which vary in size from two to several micra in length and from 0.5 to 1.0 micron in diameter. Evidently because, following cell division, the two resulting bacteria break apart sharply, the distribution of organisms in a stained smear is relatively characteristic. The individual bacilli form sharp angles with each other and have been variously compared with piles of matches, Chinese letters or cuneiform characters, to which the frequent wedge shape of the cell lends further suggestion. The occasional occurrence of true branching, which has been observed in the growth of *C. diphtheriae*, together with the irregularities of protoplasmic distribution previously mentioned, have been interpreted by some bacteriologists as tending to separate this group of organisms from the true bacteria and place it somewhat above them in the organizational scale.

The keynote of morphology in this group is the wide variation seen among strains—variation which however is confined within certain general limits. Since, as we shall see below, there are, in addition to true diphtheria bacilli, organisms which by all cultural criteria must be called *C. diphtheriae* although they form no toxin, evidently are without virulence, and also bacteria of very similar appearance, but definitely divergent growth characteristics

which are also nonvirulent, a challenge has been presented to the morphologist to attempt to differentiate microscopically between these various forms. Complex schemes have been proposed (Wesbrook et al., 1900) through which it was believed possible to differentiate nonpathogenic forms from true diphtheria, and by which the latter were separated into varying numbers of subgroups. The value of such classifications lay in the aid which it was believed they could lend to the laboratory diagnosis of diphtheria (see below). Their shortcomings stemmed from the fact that not all bacteriologists are equally capable morphologists and that, within the same strain, morphology may vary on different media and even on different batches of the same medium. The current tendency appears to be away from undue emphasis on morphology and toward the slightly slower, but more objective and dependable differentiation based on growth and biologic properties.

#### CULTIVATION

*C. diphtheriae* grows readily on most of the usual laboratory media containing "peptones" and tissue extractives. Wide differences undoubtedly occur between strains in regard to the particular amino acids, carbon and energy sources as well as accessory substances essential to growth and contributing to its speed and abundance (Mueller, 1940). Those strains which have been most fully examined lack certain synthetic capabilities in connection with the synthesis of nicotinic and pantothenic acids. Thus, nicotinic acid becomes an essential component of a suitable culture medium, as does pantothenic acid, or in some cases,  $\beta$ -alanine, from which the particular strain evolves its own pantothenate molecule. Similarly certain cultures require biotin, others make their own supply if pimelic acid is provided, whereas a further group appear independent of either substance. None of the strains which have been

studied evince requirements for so complicated a group of accessories as, for example, the streptococci do. For growth on agar plates from minute inocula, oleic acid and some further unidentified substance have been shown to be necessary (Cohen and Mueller, 1940).

**Loeffler's Medium.** The use by Loeffler of coagulated blood serum for the initial isolation of *C. diphtheriae* from cases of diphtheria resulted in the belief that such a medium manifested selective growth-promoting properties for the diphtheria bacillus, enabling it to outgrow other bacteria occurring in the throat and giving it a "normal" and typical morphology. While there is no question of the utility of Loeffler's medium for diagnostic purposes, it seems, in the light of present knowledge, that its success is due largely to the fact that it is a relatively poor medium on which the diphtheria bacillus grows reasonably well, but which is not quite good enough for the average streptococcus or pneumococcus. It is perhaps worth noting that rather widely divergent results will be obtained on Loeffler's medium, both as regards excellence of growth and morphology, depending upon the type of serum used in its preparation. Since formulae in the literature variously specify beef, sheep or swine serum, a certain lack of uniformity of results with this medium may be anticipated. Swine serum, for example, seems either to be deficient in a factor favoring the growth of certain strains of *C. diphtheriae* or more probably to contain a substance which suppresses their growth (Snyder and Mueller, 1940). The exclusive use of Loeffler's medium for cultivating *C. diphtheriae* in most laboratories probably also explains the long delay in recognizing the fact that there are at least three well-defined types of this organism which are readily differentiated by cultural characteristics, and, in a broad way, by morphology. Before further comment on cultural characteristics can be profitable, it

is therefore essential to consider the further subdivision made possible by this fact.

#### TYPES OF *C. DIPHTHERIAE*

It has for many years been known that potassium tellurite, in amounts which inhibit the growth of most bacteria, has little effect on *C. diphtheriae*. A series of tellurite-containing formulae have been proposed for diagnostic use in diphtheria, and by means of one of these, and incited by the occurrence on the continent and later in England of an outbreak of unusually severe diphtheria, McLeod, Happold and their collaborators (Anderson et al., 1931) found that it was possible to differentiate quite sharply between three types of *C. diphtheriae*. Correlation appeared to exist between the severity of the case and the types of organism found, which were consequently named *gravis*, *mitis* and *intermedius*. Although fairly general confirmation of the existence and cultural properties of these types has been achieved, it appears that there are either additional types not readily differentiated by the same criteria, or occasional atypical or transitional forms. The possibility that the three types represent dissociative phases of a single organism seems relatively remote. There is by no means uniform agreement, however, as to the correlation of type with clinical severity. This matter will receive further consideration in a later section of this chapter.

While colonies of the various types differ in appearance depending upon the tellurite formula employed, it is a simple matter to learn the differentiation with any one of the media. In general, the *gravis* colonies are largest, tend to be flat, slate gray to black in color, with a dull or matt surface. The *mitis* colonies are usually blacker, convex, with glossy surface, and somewhat smaller than *gravis*. The *intermedius* colonies are minute, pin-point in size, and vary from gray to black depending upon the medium. Colonies of *C. Hofmani* resemble



the *mitis* type in size and color but usually are sufficiently different to permit recognition by the experienced bacteriologist. Beyond the colony characteristics on tellurite plates, there are several further criteria which assist in classification. Thus, *gravis* strains ferment starch with the formation of acid, whereas *mitis* and *intermedius* are without effect. *Mitis* strains, in general, produce slight hemolysis on blood agar; *intermedius* fails to do so; *gravis* cultures give irregular results. Certain differential fea-

theria bacillus in the usual bacteriologic bouillon, the agar slope or plate, blood agar plates, etc., will vary with the type of the organism and that no further attempt at detailed description would be profitable.

#### CARBOHYDRATE FERMENTATIONS

The diphtheria bacillus typically ferments glucose, producing acid, but not gas, and fails to ferment maltose and sucrose. *Gravis* types ferment starch and dextrin, whereas

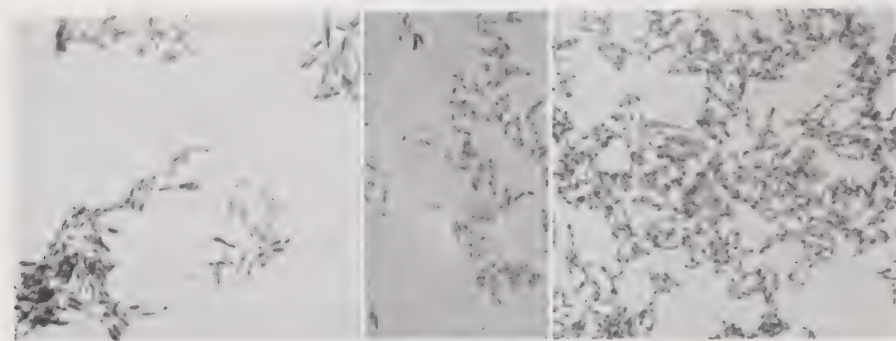


FIG. 5. Different types of *Corynebacterium diphtheriae*: (left) *intermedius*, (center) *gravis*, (right) *mitis*. Magnification,  $\times 760$ .

tures appear when the organisms are grown in broth: *gravis* strains tending to form pellicles; *mitis* to grow diffusely; whereas *intermedius* develops as a finely granular turbidity, settling to leave a clear supernatant. Morphology likewise assists in the differentiation, *gravis* cultures manifesting short forms with little metachromatic material, *mitis* occurring as long, granular organisms, and *intermedius* showing barred, club-shaped forms.

Immunologic differences subdivide the three main types still further, although a satisfactory classification is not yet achieved. The *mitis* group seems to be the most heterogeneous, whereas the majority of *gravis* strains fall into two or three groups and *intermedius* seems relatively homogeneous.

For a more detailed discussion of the types and their significance see McLeod, 1943.

It is evident from the above that the nature of growth to be expected of a diph-

*mitis* and *intermedius* are without effect on these carbohydrates. Growth and fermentation occur more promptly if the carbohydrate is incorporated into Hiss serum water than when plain broth is used as substrate. Failure to ferment should be recorded only when growth has demonstrably occurred. In starch and dextrin the absence of glucose as an impurity must be assured by suitable controls, since otherwise false positives will result.

#### TOXIN PRODUCTION

The unique property shared by all virulent diphtheria bacilli is their ability to produce, both in vivo and in vitro, the specific exotoxin causing the clinical injury in diphtheria. Nonvirulent organisms, indistinguishable in other respects, occur particularly among *mitis* strains, but infrequently, if at all, among *gravis* and *intermedius*. It is possible that nonvirulent, non-toxin-forming strains may arise through

some mutation or dissociative modification from previously virulent organisms either in the throat or in culture. The significance of toxin formation to the physiology of the bacterial cell is entirely obscure, except for very recent evidence that it may in some way be connected with the cytochrome system (Pappenheimer, 1947). A vast amount of experimentation on relative amounts of toxin formed by different strains and by the same strain at different stages of disease and convalescence, has yielded little information of value. A strain of *C. diphtheriae* isolated in 1898 by Park and Williams was found to produce rather more potent toxin than a number of other strains studied by them during the same general period, and has been generally adopted by most laboratories for routine production of toxin. Probably most of the known facts connected with toxin production stem from experience with this strain.

Foremost among the facts which developed was the irregularity of yield of toxin in different laboratories and at different times. This was undoubtedly due both to dissociative changes in the organism which took one direction during subculture in one laboratory and quite another under different conditions in a second. After 50 years, it would indeed be remarkable if any of the numerous so-called Park-8 strains in existence should prove to behave identically. The second, and equally important reason for variation in toxin production was to be found in differences in the medium employed for cultivation. Although long recognized, the phenomenon did not receive a satisfactory explanation until Pappenheimer and Johnson showed in 1936 that the concentration of iron in the medium played an important and unsuspected role in connection with toxin formation. A certain minimal amount of iron must be present in the medium in order to permit growth of the diphtheria bacillus. This is obviously due to the fact that the cell protoplasm possesses some essential iron-containing components.

It was shown that growth and toxin production increased proportionately when successive increments of iron were added to an initially deficient medium. At a concentration of about 100 micrograms of iron per liter of medium, the peak of toxin production was reached and further additions, although improving growth, resulted in a rapid fall of toxin, until at 500 micrograms of Fe per liter toxin could no longer be demonstrated. Iron concentrations in this range and frequently higher occur in the usual constituents of bacteriologic culture medium, from dirty glassware and utensils and from a variety of unsuspected sources, and it is perfectly clear that all the older statements on quantitative aspects of toxin formation are invalid, because of the failure to recognize and control this component of the medium.

Careful control of iron concentration has now made it possible to study somewhat more satisfactorily a number of factors in diphtheria toxin formation, and to show that for a particular strain of the Park-8 organism it is possible so accurately to define the chemical composition of the medium as to obtain quite reproducible yields of toxin of great potency (Pappenheimer et al., 1937; Mueller and Miller, 1940). The more important secondary factors include osmotic pressure, concentration of amino acids and of nitrogenous material, and the nature and quantity of carbohydrate required to maintain pH at a favorable level throughout the period of growth. These conditions vary from one strain to another, and it is still impracticable to compare strains and types of diphtheria bacilli as to their toxigenic capabilities in vitro in any satisfactory or really convincing manner.

Under natural conditions of growth in the throat during disease, it is still less possible to make estimations of relative toxin production (Mueller, 1941). Here the organism multiplies under conditions which differ greatly from those obtaining in vitro, and in the presence of amounts of iron consid-



erably in excess of 100 micrograms per kilo of tissue. Thus, if in-vitro conditions apply, toxin is being produced at relatively low levels. The suggestion is obvious that if one could measure toxin formation under artificial conditions duplicating those obtaining in the diphtheritic lesion, striking differences might be found to exist between strains, differences which would go far toward explaining variations in clinical severity in particular cases and in outbreaks of disease. Thus far, technical difficulties have prevented any conclusive experimentation along this line.

#### BACTERIOLOGIC DIAGNOSIS

Current views tend to place the responsibility for diagnosing diphtheria upon the clinician, leaving for the laboratory the task of bacteriologic confirmation. This practice has been adopted because of the risk to the patient of delay in the administration of antitoxin. It is safer to err on the side of an occasional needless serum treatment than to lose time which can make the difference between recovery and death. To a degree, therefore, the necessity for a very rapid diagnosis need no longer be felt by the bacteriologist, although the diagnostic method employed should be no more time-consuming than is consistent with accuracy. The method to be selected must also be readily applicable to recognition of the diphtheria bacillus in the convalescent case and the healthy carrier, as well as from the occasional unusual source such as the conjunctiva, a skin lesion or wound diphtheria.

The diagnosis depends upon the recognition of a diphtheria bacillus in material taken from the site of infection, usually the throat. It is important that the specimen be carefully obtained, and that it represent material from the membrane, if present, and from the area of inflammation. It should be taken by the physician with good illumination of the area and sent to the laboratory

with the least possible delay. While the diphtheria bacilli are not especially delicate, and may survive for many hours on a cotton swab, they die out progressively, and the interval between taking the culture and its examination should be no greater than necessary. In the laboratory, a Loeffler slant, a blood plate and a tellurite plate are inoculated with the swab, and a smear is prepared which may be stained with dilute fuchsin and examined for the organisms of Vincent's angina. It is not recommended that any attempt be made to identify *C. diphtheriae* in the direct smear. Except in the hands of a bacteriologist of long experience in such diagnosis, the chance of error in either direction is too great. After 15 to 24 hours' incubation the cultures may be examined—the blood plate for hemolytic streptococci, and the tellurite plate for the gray or black colonies of diphtheria bacilli. Frequently, no growth whatever will be found on the tellurite plate. Provided the blood plate has shown the presence of viable bacteria on the swab, this provides strong presumptive evidence that no diphtheria bacilli are present, but the plate should be reincubated for 24 hours more and again examined before being reported finally as negative. In examining the tellurite plate one must keep in mind the difference in appearance of the three types of *C. diphtheriae*, and especially the relatively inconspicuous nature of *intermedius* growth. If colonies are present which suggest any of the types, a smear stained with methylene blue will enable one promptly to determine whether or not they are corynebacteria of some sort. If so, their morphology, together with the colony appearance will frequently make identification quite certain. In case of doubt, the somewhat different morphology of the organism on the Loeffler slant may be of assistance. *C. Hofmanni*, for example, is sufficiently characteristic to make recognition relatively easy.

Where considerable numbers of cases of infection due to a single type of *C. diph-*

*theriae* are occurring in a community, the matter need be carried no further; the bacteriologist will readily identify the strain and a final report may be made covering the presence of the diphtheria bacillus, the hemolytic streptococcus and Vincent's angina. The suspected individual case, the possible carrier (except during an epidemic of a known type), perhaps the occasional refractory convalescent carrier, and organisms from any unusual source, such as a skin lesion, should be isolated for further identification, and a virulence test must be carried out. It is usually desirable to restreak a second tellurite or blood-agar plate from the growth on the initial tellurite plate, and then to isolate the culture from a single colony to a Loeffler slant or other suitable medium. The pure culture thus obtained is used to inoculate tubes of Hiss serum water to check the fermentative properties. Ordinarily glucose, sucrose and starch will be found to be sufficient for this purpose. A tube of nutrient broth and a blood plate (if necessary) may also be inoculated and will yield additional information regarding type. Except for a special study, no agglutinative classification need be attempted.

The virulence test is carried out in the following manner. The growth on a Loeffler slant is emulsified in three or four cubic centimeters of broth to make a fairly heavy suspension, and from 0.1 to 0.2 cc. is injected intracutaneously on the shaved side of each of two guinea pigs, one of which has received 500 units of diphtheria antitoxin intraperitoneally on the previous day. In case of necessity, rabbits will be found equally suitable. The unprotected animal should be given from 30 to 50 units of antitoxin intraperitoneally about three or four hours after the injection of the culture. This prevents premature death without interfering with the development of the skin lesions. By this method it is possible to test several cultures on one pair of animals and since one may and should include a culture of known virulence as a control on the suita-

bility of the animals, it has a definite advantage over the older procedure of subcutaneous injection of a single culture with death of the test animal as evidence of positive virulence. Inflammatory lesions begin to develop at the sites of injection of virulent strains in 24 hours and continue to definite necrosis in 48 to 72 hours. The control animal should show a completely negative response and is used to exclude any effect due to an agent other than diphtheria toxin.

#### PATHOGENICITY FOR ANIMALS

Natural infection of the lower animals with *C. diphtheriae* appears not to occur. A variety of experimental animals, however, are susceptible to the effects of its toxin, and it has been possible to establish with the organism experimental infections which simulate the human disease. Thus, Loeffler, in his early experiments was able, by intratracheal inoculation, so to infect rabbits and pigeons as to obtain typical diphtheritic pseudomembranes at the site of injury. Indeed similar results had earlier been observed following inoculation of these animals with infectious material taken directly from human lesions (Wilson and Miles, 1946b).

One of the characteristic features of bacterial toxins is a marked variability in degree of action on different species of animals. There is almost always a well-marked selectivity evidenced by a relative susceptibility of certain species and the comparative resistance of others. Within a single species, however, and excluding specific antitoxic immunity resulting from experimental procedure, susceptibility is generally much more uniform, and the more nearly one approaches a pure blood line of animals, the more regular the effect. Thus, with diphtheria toxin the susceptible human being and the rabbit are perhaps the most exquisitely sensitive. The guinea pig is also highly susceptible, and horses, dogs, cats.



monkeys and birds are all affected in varying degree. The rat and mouse are highly resistant. As much as 200 or 300 times the lethal doses for the guinea pig is required to kill mice, which means that per kilogram weight perhaps 25,000 times as much toxin must be required to interfere with vital function in these animals as in a child. The explanation for this species difference is unknown. It is not due to antitoxin (Coca, 1921), and must strike deeply into the question of site and mode of action of the toxin molecule. An understanding of the matter could well lead to practical results of great value.

Injection of diphtheria toxin subcutaneously into an animal such as the guinea pig is followed in a few hours by local swelling and apparent tenderness. Death ensues in from 24 hours to several days, depending upon the amount of toxin given. With suitably gauged sublethal doses, late paralyses may occur resembling those seen in man during convalescence from diphtheria. Examination at autopsy following death from a fatal dose reveals intense edema of the subcutaneous tissue at the site of injection, often hemorrhagic in character. Beyond this, the most constant and striking pathologic picture is the marked congestion of the adrenal cortices, frequently accompanied by hemorrhage. Occasionally hemorrhage in the pericardium or diaphragm occurs, and the heart muscle, liver and kidneys may show fatty degenerative changes.

If cultures of virulent diphtheria bacilli are injected, rather than toxin itself, the chain of events and the pathologic findings follow closely on those observed after the administration of the toxin. The bacilli themselves, as Loeffler recognized, remain localized and, in general, are found at autopsy only in the vicinity of the site of inoculation. Positive cultures occasionally obtained from the viscera appear to be due to terminal or post-mortem invasion, rather than attributable to any active invasive ability on the part of the organism.

In the diphtheria bacillus, therefore, we have an organism possessing in some degree the ability to survive and to establish itself in the healthy tissues of a susceptible animal to an extent sufficient for the elaboration of an amount of toxin which is capable of causing death of the animal through specific and distant action on certain kinds of tissue. This property, common to all "virulent" strains of *C. diphtheriae*, is lacking in "non-virulent" strains which may otherwise be indistinguishable from the former group. It is perhaps not clear whether the failure is due to inability to survive in the tissue or to a difference which eliminates the toxin-forming function. One assumes that such organisms would also fail, *in vitro*, to produce toxin, but in view of the difficulties surrounding estimation of toxin formation in the test tube, to which allusion has already been made, the question may still be considered open. It is well to bear in mind, however, that the result of a "virulence" test must depend upon at least three factors, first the amount of preformed toxin in the inoculum, second, the degree to which the injected organism grows in the tissue, whether it be cutaneous or subcutaneous, and, third, the rate at which it elaborates toxin during that growth. A fourth factor may enter the picture if certain organisms produce something akin to "spreading factor" or an "aggressin," which, in addition to the toxin molecule, assists them in becoming established. Efforts have been made to separate these various effects experimentally, but thus far, without convincing success. Until such time, then, as the "virulence" test can be broken down into its component parts, it must be kept in mind that it represents a summation of toxigenicity and invasiveness and is susceptible to very little quantitative measurement. When methods have been devised adequately to estimate and relate these factors, we shall probably be able to obtain direct evidence on the relation of type and

strain to clinical severity of diphtheria in man.

#### TYPES OF *C. DIPHTHERIAE* IN RELATION TO CLINICAL SEVERITY

The *gravis* type of *C. diphtheriae* was at first believed to be responsible for the unusually severe and highly fatal outbreak of diphtheria which appeared in Germany in about 1927. Since type differentiation was not established until four years later by McLeod and his colleagues (Anderson *et al.*, 1931), it is not entirely certain that such was the case. Present evidence indicates that diphtheria in epidemic form is frequently due to the *gravis* type, with *intermedius* occupying the position of next importance; *mitis* is found most frequently in endemic areas. The first two types tend to produce the more toxic forms of the disease, with a correspondingly greater mortality than *mitis* infections. Fatality with the *mitis* strain is believed to be more often due to tracheal or laryngeal obstruction than to toxic injury. For a thorough discussion of the matter, McLeod's review (1943) should be consulted.

#### PATHOGENESIS IN MAN

The diphtheria bacillus occurs in nature, so far as is known, only in lesions of the specific disease in man, and in the throats and noses of the normal human carrier. From one of these two sources, a virulent diphtheria bacillus reaches more or less directly—by droplet, contact or fomites—the throat of a susceptible individual. Growth presumably is initiated in a superficial layer of mucus and desquamated epithelial cells and small amounts of toxin are elaborated. This toxin, absorbed into adjoining living cells, destroys them in a few hours through its local necrotizing action. The nidus of necrotic tissue supplies favorable conditions for further growth of the organisms, more toxin is formed and the process extends

both laterally and more deeply into the tissue.

Meanwhile there is an inflammatory reaction on the part of the body, capillaries engorge, leukocytes enter, red cells become extravasated and a layer of exudate begins to form which is composed of all these various elements. This is at first grayish and inconspicuous, but as the process continues it soon becomes thicker and tough, forming a dull white layer or "pseudomembrane" covering the area. The initial lesion may cover a tonsil or a portion of the posterior pharynx. In some cases it is limited to the posterior nares, or the trachea and thus may elude observation. At this stage of the disease, the patient has typically a moderately sore throat lacking the acutely inflamed appearance, swelling and pain of a streptococcus infection and has a relatively low fever of from 100° to 102°. He usually manifests, however, a degree of prostration out of all proportion to the fever and visible difficulty in the throat. The membrane may spread with considerable rapidity over the tonsils, uvula and posterior pharynx. The dull white color gives place to a dirty gray and later to brown or, in some instances, black as a result of hemorrhage. Separation of the membrane by mechanical means during the early stages uncovers bleeding points and is rapidly followed by the formation of fresh exudate.

The cervical glands early become swollen and tender, and in the severe, or "bull-neck," variety there is a massive edema of the tissue of the neck and chest. If the membrane developed initially in the larynx, or if it extends to that site and continues further into the trachea, death may result from mechanical stoppage of the air passage unless promptly relieved by intubation or tracheotomy. Excluding such mechanical termination of the infection, and in the absence of antitoxin treatment, the patient will run the natural course of the disease and either die during the acute stage as a result of general toxic effect, succumb after



a somewhat longer time as a result of cardiac damage by the toxin, or recover after perhaps showing evidence of neurotoxic injury such as paralysis of the soft palate, ciliary muscles of the eye or the extremities. The membrane separates after a few days and is eliminated, leaving as a rule very little ulceration of the underlying tissues.

The infection may occur initially in the ear, or in the anterior nares, and in the latter site particularly is likely to be relatively mild, though prolonged. Rarely, the conjunctiva may become the site of an extremely severe and destructive diphtheritic lesion.

Skin or wound diphtheria is rarely seen under ordinary conditions in temperate climates. In the tropics, however, it is apparently not uncommon and indeed presented the armed forces with a rather serious problem in Africa and in portions of the Pacific theatre during World War II. The infection appears usually at the site of some relatively minor injury, a bruise, scratch or blister and develops as an ulcer, showing little tendency to heal, with sharply demarcated edges and a dirty grayish slough or membrane covering the base. Such infections may last for weeks or months and cover areas of several centimeters diameter. Their nature may be unsuspected because of the rarity of the condition under normal circumstances and in temperate climates. Conflicting reports are recorded regarding the type of flora and the virulence status of the diphtheria bacilli obtained in culture, but the consensus of opinion appears to be that virulent organisms of the *mitis* type are generally involved. Absorption of toxin is apparently minimal, since few fatalities, although a not inconsiderable number of late paralyses, have been observed. Response to antitoxin seems not to be uniformly favorable, and it is possible that the etiology of the condition is complicated by some other factor.

The pathology of the fatal human case of diphtheria, except for the more obvious

local picture, is not particularly striking. Evidence is seen grossly of toxic degenerative change in heart, liver, kidneys and adrenals, which consist microscopically of parenchymatous degeneration, fatty infiltration and necrosis. It is not possible, on the basis of these observations, to reach any conclusion as to specific sites of action on the part of the toxin. The cardiac damage, one of the very common causes of fatal termination, was for a time believed to be due to vasomotor disturbances in circulation, but it now appears that actual injury to cardiac muscle, sometimes involving the conducting mechanism, may equally well be involved. The lesions in the adrenals are much less conspicuous than in the guinea pig, but MacCallum states that these organs are likely to show more prominent degenerative changes than the other viscera.

#### TREATMENT

Since diphtheria is essentially a toxemia with very little invasion of the tissues by the organisms themselves, it appears that prompt recovery should follow administration of the specifically neutralizing antitoxin. The problem, unfortunately, is not quite so simple. Diphtheria toxin in the test tube, it is true, is neutralized promptly, completely and multiple for multiple by antitoxin. Although it can be shown that the toxin is not actually destroyed, the union is a relatively firm one, and dissociation apparently does not occur to a significant degree if the complex is injected into the animal body. Free toxin, however, when introduced into the circulation evidently attaches itself promptly and firmly to some of the body cells. It is not known whether the ability so to combine with toxin is an attribute of a certain cell type or of some specific component common to various cells. In any event, when this union has taken place, the toxin seems no longer to be subject to neutralization by antitoxin and the train of events which the toxin is

capable of initiating continues, even in the presence of a considerable excess of antitoxin in the circulation. Thus, if a series of animals be injected with similar quantities of diphtheria toxin, the animals being divided into groups and varying amounts of antitoxin administered immediately to one group, after five minutes to a second group, and so on, it is found that the amount of antitoxin necessary to save life increases very rapidly with the time interval. The following tabulation, cited by Zinsser *et al.* (1939) shows the amounts of antitoxin necessary to prevent death in rabbits, at various intervals after the administration of ten fatal doses of toxin. It is evident that after a delay of 90 minutes, no amount of antitoxin will save the animals.

Given after 10 minutes.....	5 units antitoxin
Given after 20 minutes.....	200 units antitoxin
Given after 30 minutes.....	2,000 units antitoxin
Given after 45 minutes.....	4,000 units antitoxin
Given after 60 minutes.....	5,000 units antitoxin
Given after 90 minutes.....	No amount

The exact explanation of the failure of toxin to be neutralized by antitoxin after attachment to the tissues is not yet known. There are, of course, several possibilities. Perhaps the antitoxin is unable to enter the body cells into which the toxin may pass. The toxin may combine with the cell by the same bonds with which it unites with antitoxin; these being satisfied, there is nothing left to bring about the union. Again, the toxin may become altered in some way following union with the cell, and the altered molecule may not be able to combine with antitoxin. To extend this possibility, the altered toxin molecule itself may be the injurious agent, rather than the original toxin molecule. Perhaps, finally, the toxin brings about some change in the cell almost as soon as it becomes attached, and the injury being accomplished, it does not matter that the toxin molecule is then neutralized. Whatever the ultimate explanation, and, as pointed out in an earlier section, its understanding may well be of great significance,

the fact itself supplies the guiding principle which must be applied in antitoxin therapy. *Treatment must be prompt and adequate.*

The physician seeing a patient whose throat suggests the reasonable probability of diphtheritic infection should send a culture to the laboratory, but should at once administer antitoxin. The laboratory report will later confirm or refute the diagnosis, but far less damage will be done by the administration of an occasional unnecessary dose of antitoxin than by delay in its use when it is required. The following tabulation of fatality according to the day of the disease on which antitoxin was administered is quoted by Russell (1943).

TABLE 21

ANTITOXIN GIVEN ON	CASES	CASE FATALITY
1st day of disease.....	225	0
2nd day of disease.....	1,441	4.2
3rd day of disease.....	1,600	11.1
4th day of disease.....	1,276	17.3
5th day of disease and upwards	1,645	18.7

No definite rules can be laid down for the amount of antitoxin required for adequate therapy. The actual amount of toxin which has gained entrance to the tissues on the first or second day of the disease is probably small, and could be expressed in terms of a relatively few guinea-pig minimal lethal doses. Were this not so, the mortality in untreated diphtheria would be 100 per cent. Since one unit of antitoxin will neutralize (in the test tube) about 30 M.L.D., it might appear that from 100 to 1,000 units would be more than adequate to obtain the desired effect. However, since it is known from experiments such as those mentioned above, that within limits, increasing the antitoxin will save life, even after contact of the toxin with the tissue, there is a reasonable basis for the current practice of treating diph-



theria with relatively large doses of serum. The objective should be to counteract as much as possible of the injury already done, and to prevent further absorption of toxin. Antitoxin is eliminated slowly, so that a single large dose, raising the blood level as high as possible, will assure the maximum immediate therapeutic effect and provide a level in the blood for many days which will be adequate to cope with further toxin as it is absorbed slowly from the local process in the throat.

Various figures for the recommended unitage of antitoxin are to be found in the literature. One may perhaps state the general average as suggesting 100 units per pound body weight in mild cases, up to five times that amount in the severe forms, as adequate therapy. It should be administered in a single dose, intravenously except in the very mild case, where intramuscular injection is satisfactory. Skin tests for sensitivity to the protein of the antitoxin preparation must of course be carried out, and where necessary, desensitization must be undertaken, or bovine antitoxin may be employed. In general, it appears that there is little risk of anaphylactic difficulty with the purified and "despeciated" antitoxins now generally available (see Antitoxin).

#### THE DIPHTHERIA CARRIER

The organisms commonly disappear from the throat after a variable period of time following recovery from an attack of diphtheria. This is not invariably true, however, and positive cultures may be obtained in some instances for months after convalescence. The treatment and disposition of these chronic carriers becomes, then, a most troublesome problem, both scientifically and administratively, and one for which no satisfactory solution has been found. In many communities the health regulations require a continuation of quarantine of the individual until negative throat cultures are obtained on three consecutive days; as a re-

sult, considerable hardship is suffered by the patient, and the hospital also may find itself in a difficult situation. Experience has shown that surgical correction of nose and throat abnormalities, tonsillectomy, etc., coupled with mild gargles and mouth washes will in some instances result in clearing up the condition. The use of bactericidal sprays and washes has been without effect. At present, evidence is accumulating that combined treatment of the case with antitoxin and penicillin, in which administration of the latter is continued for a time into convalescence, leads to a much more rapid disappearance of positive cultures than was formerly true. Opposed to this is the fact that some observers find diphtheria bacilli reappearing in the throat within a few days after interrupting the penicillin therapy. It is therefore still premature to assume that a permanent cure for the convalescent carrier problem has been achieved.

The question is occasionally raised as to whether the diphtheria bacilli gradually lose virulence in the course of convalescence, and through dissociation or in some other manner become transformed into harmless saprophytes. From time to time attempts have been made to approach this possibility experimentally, and a certain amount of evidence, not altogether convincing in character, has been found to support such a view. Certainly one cannot anticipate the occurrence of such losses of virulence with regularity, for there are many instances of known infection resulting from exposure to released convalescents.

#### IMMUNITY AND EPIDEMIOLOGY

In its basic features, immunity to diphtheria is probably the simplest concept in the whole field of the study of specific resistance to disease. Unfortunately, in certain details of theory as well as of application, difficulties are encountered.

In the absence of previous contact with the diphtheria bacillus or its toxin (exclud-

ing the passive transfer of antibodies from immune mother to foetus through the placenta) the human infant possesses no neutralizing ability for diphtheria toxin and is therefore susceptible to infection when brought in contact with the diphtheria bacillus, and presumably would retain this state of susceptibility indefinitely into later life. One might postulate a type of non-specific resistance which could be effective in certain individuals under these conditions, such as a particular bacterial throat flora which provided unfavorable conditions for the growth of diphtheria bacilli should they find their way to it. This sort of a mechanism, though never demonstrated, may occasionally exist but has no relation to the type of immunity under discussion. Excluding some nonspecific protective mechanism, infection of such an individual with a virulent diphtheria bacillus would quite surely result in an attack of clinical disease. If the organism were of high virulence, and specific treatment were not available, the individual would die within a few days, since even under the most favorable conditions the body probably cannot initiate effective antibody formation in less than a week. If, however, the virulence of the infecting strain were only moderate, a nonfatal illness might result; and in the case of a practically nonvirulent organism, perhaps no discomfort whatever would be experienced. In either of the two latter events the tissues would have experienced contact with diphtheria toxin, although in extremely small quantity. A single exposure to minute amounts of toxin extending over a period of a few days might serve as a sufficient stimulation to the tissues to form antitoxin in quantity which could be detected experimentally and which would render the individual immune, at least for the time being, to further infection. Much more probably, it would result in nothing more than a "conditioning" of the tissues in such a way that later contact with diphtheria toxin would cause a much

more effective antitoxin response to take place.

Two points should now be evident: (1) recovery from an attack of diphtheria need not result in immunity, either permanent or transient and (2) repeated encounters with organisms of low virulence, without evidence of disease, may result in a fairly solid state of specific resistance to infection with a virulent strain. Before the advent of antitoxin, and in communities where diphtheria was endemic, with periodic occurrence of epidemics, this probably represented in a general way the picture of the immunity in the population. Diphtheria was primarily a disease of children, and there was a fairly effective level of immunity in adults. Therapeutic antitoxin perhaps did little to alter this picture except to prevent a considerable number of deaths which otherwise would have occurred.

It must be kept in mind that the above course of events takes place only because diphtheria bacilli of all grades of virulence are constantly present in a significant proportion of the population, and diphtheria infection is relatively common. If diphtheria were to disappear from a community, the dissemination of virulent organisms from case to contacts would cease. If one assumes the parent type to be the virulent form from which organisms of lesser or no virulence develop by a series of dissociative changes, then these degraded forms too might be expected to become rare or perhaps eventually to vanish, and, lacking the stimulus for further antitoxin formation, such a community would, over the years, lose its status of adult immunity and become uniformly susceptible at all age levels.

Artificial immunization of populations against diphtheria may be an important factor in bringing about such a reduction in the prevalence of diphtheria bacilli, but one must not overlook the possibility that a natural downward trend of disease such as occurs with many types of infection could also accomplish the same thing. Immuniza-



tion, of course, introduces perfectly definite changes into the normal chain of circumstances. Relatively high levels of antitoxin can readily be induced in the majority of infants by two or three injections of toxoid. This antitoxin persists for a variable period, depending on factors in the child which cannot yet be defined. Duration of the immunity may be only a few months but in most instances it will be a few years. In the absence of further artificial stimulus to immunity, natural factors again become operative, and the immunity may be expected to continue if diphtheria bacilli are abundantly present in the environment, otherwise to disappear. Hence, the more successful the campaign to stamp out diphtheria, the more certain it becomes that a susceptible adult population will develop if one depends on immunization only during infancy. Clearly, it is imperative to add to the immunization program two or more "recall" or "booster" injections in later years in order to avoid future difficulty.

It is evident from the above discussion that within a population in which diphtheria is prevalent, it is incorrect to think of the existence of two simple groups, the susceptible and the immune, since the one merges into the other through an infinite series of gradations. Those with no previous contact will constitute one fairly sharp group of high susceptibility, whose blood, upon examination, would show no antitoxin. Exactly the same laboratory findings would be manifested by others whose previous contacts with the diphtheria, though definite, were minimal, and by others with more extensive contact, who, because of lapse of time or personal idiosyncrasy, had either lost or failed to produce antitoxin. The majority of individuals would possess measurable amounts of antitoxin, i.e., more than 0.001 unit per cc. and extending upward possibly to many units, especially if artificial immunization had been employed. Clearly, then, the result of exposure of an individual to infection within such a group

would depend upon a balance between several factors, including at least the virulence of the organism, the degree of exposure (size of the dose), and the unique immune status of that individual. To these should perhaps be added other less well-defined "predisposing factors," such as the general well-being of the individual, the kind of normal throat flora present, and others (see also Dudley, et al., 1934).

#### THE SCHICK TEST

Introduced by Schick in 1913, this test depends upon the fact that diphtheria toxin exerts a local destructive or irritating effect upon tissue. If the blood passing through the tissue contains antitoxin, it neutralizes the toxin and no injury occurs, otherwise a visible reaction develops, the severity of which parallels the amount of toxin injected. Toxic filtrates of the diphtheria bacillus invariably contain, in addition to toxin, other products of growth which may of themselves produce injury in some individuals. These materials, probably protein in nature, will withstand a moderate amount of heat, sufficient to inactivate the highly heat-labile toxin. It is, therefore, customary to include a control in carrying out the Schick test, in order to exclude this type of response. The materials used are the following:

(1) Diphtheria toxin so diluted that  $\frac{1}{50}$  M.L.D. is contained in 0.1 cc. of the solution. Because of the extreme lability of the toxin, the nature of the diluent is of considerable importance, since substances such as certain commercial peptones, which are highly effective in preserving the activity of the toxin, can occasionally induce severe local and even general symptoms of anaphylaxis in certain sensitive individuals. The Massachusetts Antitoxin and Vaccine Laboratory now employs a borate buffer solution containing human albumin. Others have employed gelatin as the stabilizing material.

(2) The same material heated to 60° C. for 15 minutes.

The test is carried out by a careful intradermal injection of exactly 0.1 cc. of the dilute toxin on the flexor surface of the fore-arm, and a similar injection of the heat-inactivated material on the opposite arm. Occasionally a slight degree of edema appears around both injection sites within a few minutes, but shortly disappears. No significance attaches itself to this phenomenon. The injected areas should be inspected at 24 or 48 hours, and again at the end of a week, and the following types of reaction can be distinguished.

**Positive.** At the site of toxin injection an area of redness begins to appear at about 24 hours, and becomes progressively more pronounced until it reaches a maximum in about a week. At this time it covers an area up to 3 cm. or somewhat more in diameter and may show moderate swelling and slight tenderness. There is usually a smaller, more deeply colored central area 1.0-1.5 cm. in diameter, dark red in color, which a few days later turns brown and eventually desquamates, sometimes leaving a slightly pigmented surface which may persist for some time. The control arm remains completely negative throughout. Such a positive test indicates very little or no circulating antitoxin and probable susceptibility to diphtheria. The reaction is shown in Plate 1.

**Negative.** Both arms remain without reaction of any sort. Antitoxin is present in reasonable amount, sufficient to supply immunity to an ordinary exposure to diphtheria. Observations indicate that this level is between  $\frac{1}{30}$  and  $\frac{1}{100}$  of a unit per cc.

**Pseudoreaction.** An area of erythema appears equally on both arms beginning at about 18 hours, increasing to 24 or 36 hours and then gradually fading at both sites alike, to disappear completely within two or three days. Such individuals possess adequate antitoxin to confer immunity, but are, in addition, allergic to some component of the injected material (see Plate 1).

**Combined Reaction.** Commencing like the pseudoreaction, the reaction in the control arm follows the course described for that type of response, whereas at the point of injection of the toxin, the reaction passes through the successive stages of a positive test. In such cases there is evidence of susceptibility through lack of antitoxin, but also of the existence of allergy as with the pseudoreaction. The current tendency is to interpret this as indicating previous contact with the diphtheria bacillus or its products, and as evidence of at least a potential state of immunity. These reactions are seen most frequently in adults and present a problem for which no completely satisfactory solution has yet been found, since attempts to increase their antitoxin levels with toxoid are frequently followed by severe systemic reactions as a result of the allergic state. The matter will be considered more fully below.

An additional control sometimes employed in carrying out the Schick test in adults and which may, if desired, replace the usual Schick heat-inactivated control, is the Moloney test (Moloney and Fraser, 1927). This consists in the intradermal injection of 0.1 cc. of a 1 to 10 dilution of fluid diphtheria toxoid. It leads, essentially, to the development of an accentuated pseudoreaction, and may often give such a reaction when the usual Schick control does not do so. This of course results from purely quantitative considerations, since the amount of bacterial products contained in 0.1 cc. of 1 to 10 toxoid may represent as much as 1,000 times the quantity present in the  $\frac{1}{50}$  M.L.D. Schick dose.

#### ARTIFICIAL IMMUNIZATION

There are three types of preparation which may be successfully employed for active immunization of an individual or of a population.

**Toxin-Antitoxin.** This consists of a nearly neutral mixture of the two compo-



nents. Various amounts of toxin have served as the basis for the immunizing dose at various times, and the tendency has been toward the use of smaller, rather than larger amounts. It is now pretty well agreed that 0.1 L+ unit of toxin is adequate. Actually, there are several considerations which make this substance the least desirable of those available for wholesale immunization. The antitoxin portion is horse protein, and there is the potential danger of inducing sensitization or of eliciting anaphylaxis in a previously sensitive person. Further, it is possible that the toxin may dissociate itself from the antitoxin—for example, following accidental freezing—and thus lead to severe toxic manifestations when the mixture is injected. Finally, the amount of antigen is materially less than can safely be administered in the form of toxoid, and it may, therefore, be somewhat less effective. For these reasons, its use has now given place almost universally to toxoid.

**Fluid toxoid** consists essentially of a filtrate (Berkefeld, Mandler, or Seitz) of a well-grown broth culture of the diphtheria bacillus, to which has been added from 0.3 to 0.5 per cent formalin, followed by incubation at 37° until toxicity has disappeared. A small amount of antiseptic such as merthiolate is added to prevent accidental contamination. Toxoid is administered in doses of 0.5 or 1.0 cc. containing perhaps between 15 and 100 flocculating units. Usually 3 doses at intervals of three weeks are employed.

**Alum precipitated toxoid** represents a suspension of the precipitate obtained by adding alum under defined conditions to fluid toxoid. In its preparation, a certain amount of inert and perhaps objectionable material present in the crude toxoid is eliminated. In addition, the presence of alum and insoluble material appears to hold the antigen in the tissue at the point of injection and to increase the antigenic potency of the toxoid. Experience has shown that

two doses a month apart are usually adequate.

**Fluid Toxoid vs. Alum Precipitated Toxoid.** Each of these materials has its proponents, and the choice of one or the other is largely an individual one. Alum toxoid is somewhat the better antigen of the two because of the local stimulating effect of alum on the tissues. Thus, it is possible to accomplish with two doses of this material an objective requiring three injections of the fluid form. This very intensification of antigenicity may, upon occasion, become a disadvantage, for it may lead to antibody production against some nonspecific component of the medium which would otherwise be too weakly antigenic to have an effect. Thus, unpleasant reactions could be encountered upon later injection. Moreover, the localizing and irritating effect of the alum may be great enough in some cases to lead to tissue breakdown and to the formation of sterile abscesses. Since fluid toxoid is known to be entirely effective, one must balance the advantages of two injections rather than three against a somewhat increased probability of unpleasant after-effects.

#### CONTROL OF DIPHTHERIA

Information which should make it possible to reduce diphtheria to a minimum within a community is now available, although optimal procedures for the adult population are not yet clearly defined. Immunization of infants should be done at the age of a few months, using either fluid or alum precipitated toxoid. For convenience, this toxoid may be combined with whooping cough vaccine and tetanus toxoid. It is well to point out that the traditional passive immunity of the newborn child against diphtheria, which lasts for a few months, can occur only if the mother is immune, and that currently the various forces discussed above have in many communities led to a state of affairs where this is only

true in a relatively small proportion of instances. It should also be noted that the belief is prevalent that the newborn child responds badly to vaccination because the antibody production mechanism does not begin to function well for some months. Direct evidence as to the earliest age at which one may confidently proceed with immunization seems to be wanting.

A "booster" dose of toxoid should be administered at school age, and it would undoubtedly be more effective if two such injections were given: one at three years, the second at six. These immunizations should be universal, and no Schick testing need be done. They should result in a reasonably immune population up to adolescence. From this point the matter becomes more complex. Allergy to components of diphtheria toxoid occurs in an increasing proportion of the population at the same time that antitoxic immunity may be disappearing. A Schick test and a Moloney test upon entering secondary school would define the requirements in a large group of a community. These injections would quite certainly also stimulate marked antibody response in a great many who had received the earlier immunizations described and will frequently be sufficient in themselves to "reverse" a positive test. If a second Schick test could be done after an interval of two months on those initially giving positive tests, a fair proportion, perhaps the greater part, would be found to have reversed. Those who were still positive, particularly if they also show a positive Moloney test, present the problem which will continue to be encountered on into the adult age groups.

The reactions to diphtheria toxoid in adults are probably never fatal, but they are sufficiently intense to interfere seriously with any wholesale immunization plan. One sees all grades of response, from local tenderness and swelling, to severe generalized illness with high fever and complete incapacitation lasting for two or three days. Formerly believed to be due to sensitivity

to bacterial proteins other than toxin, recent investigations by Pappenheimer, Jr. (1948a), using toxoid prepared from highly purified toxin as well as formalinized non-toxic bacterial proteins, indicate that in many instances the allergy is directed to the toxoid itself, and that it will not be possible to avoid the difficulty by using a toxoid from which bacterial protein has been eliminated. One cannot outline a definite program, therefore, to protect adult populations. The procedure chosen must be adapted to the situation. If an individual, such as doctor or nurse who must come into contact with diphtheria, is to be protected, a judicious use of Schick tests and intradermal toxoid, supplemented by small subcutaneous injections of toxoid, will probably accomplish the purpose. For large groups, for example in the armed forces or in a community, one must balance the various risks and proceed by the best plan which is administratively feasible according to the facts discussed above.

#### DIPHTHERIA ANTITOXIN

The production in horses of diphtheria antitoxin of relatively high titer, for use in the therapy of diphtheria, has been carried out in this country since 1895. Before the introduction of toxoid by Ramon, in 1924, considerable difficulty attended the process of immunization because of the great risk of toxic injury to the horses. This can now be avoided, in a great measure, by the substitution of toxoid for toxin at all stages of the immunization program. It is not our purpose to present a detailed schedule of immunization, since this varies among producers themselves. In general, the plan is to inject repeatedly at intervals of a week or somewhat longer, with gradually increasing amounts of the toxoid or with active toxin.

Horses vary, as indeed do all animals, in their individual response to an antigen. It is, therefore, becoming customary to give a single



injection or short course of injections to a considerable number of horses, and wait for some months. After this conditioning, a single stimulating dose is given, and a blood sample taken 10 days or two weeks later. On the basis of titers of antitoxin found in these sera, the required number of horses are selected for more intensified immunization. After a number of injections and when a sufficiently high titer is attained, 9 liters of blood are taken with sterile precautions from the external jugular vein of each horse. A further injection of toxoid is given, and in two weeks more blood is removed. This series of alternate injections and bleedings can be maintained often for many months, until the horse becomes exhausted through anemia. Often a period of rest will make further antitoxin production possible in such animals.

The blood obtained in this way is collected in citrate solution to prevent clotting. The plasma is removed after the red cells have settled out, and the antitoxin, which is present in the pseudoglobulin fraction, is usually concentrated to remove inert protein and other materials. Older methods employed fractional precipitation with ammonium sulfate, followed by dialysis, while current practice frequently combines such a procedure with partial peptic digestion (Parfentjev, 1934; Pope, 1939) resulting in still further removal of the species-specific protein likely to induce anaphylactic shock in sensitive individuals. Some producers effect a still further purification by means of ultra filtration rather than dialysis, thus eliminating water along with salts and yielding a preparation of correspondingly high unitage.

The antitoxin content of the final product is estimated in terms of arbitrary units, the value of which is entirely empirical and varies in different countries. The United States unit is maintained by the National Institute of Health, which supplies carefully standardized serum to producers on request. By definition, one unit of antitoxin injected into a 250-gram guinea pig together with a quantity of diphtheria toxin called L+ dose, (L = *limes* or threshold) results in death of the animal in four or five days. The standard antitoxin is therefore used to establish the L+ dose of a batch of toxin, and the amount of toxin so determined is

mixed with varying dilutions of the antitoxin to be tested, a series of guinea pigs injected, and the unit thus measured. A rapid approximate estimation of an unknown toxin or antitoxin can be obtained by the Ramon flocculation method.

## OTHER CORYNEBACTERIA

It has already been pointed out that organisms which are grouped in the general category of *corynebacteria* are widely distributed in nature. Available evidence fails to implicate any of these with actual pathogenic processes in man, although a few may induce disease in certain animals. For the bacteriologist concerned primarily with human disease these organisms are important to the extent that they frequently confuse the diagnosis of diphtheria, and render obscure the significance of other types of culture in which they are frequently encountered. Experience has shown that one should exercise great conservatism in drawing conclusions as to the relationship of these organisms to disease process, since their widespread occurrence is prone to result in accidental contamination.

### C. HOFMANNI

This organism is often found in the throat, either healthy or diseased, where it lives as a harmless saprophyte. It grows readily on the usual laboratory media, including Loeffler's medium and tellurite agar. Because of the frequency with which it occurs, it is the most troublesome of the diphtheroids in connection with the diagnosis of diphtheria. As seen in stained smears, the Hofmann bacillus is usually somewhat shorter and more plump than *C. diphtheriae*, and exhibits a tendency to bipolar staining, without the appearance of metachromatic granules. The trained observer usually recognizes it without difficulty, but assurance comes only with extensive experience. On the tellurite plate,

colonies of *C. hofmanni* may resemble closely those of *mitis* strains of the diphtheria bacillus. Here also, experience aids in differentiation, the saprophyte often forming a colony with a rather wide grayish or white margin and a dark central portion, whereas the pathogen tends to produce a more uniformly dark or black colony. The modification of Lev (1947) permitting the detection of glucose fermenting colonies on tellurite agar should prove valuable. Since there are known to be various serologic types of Hofmann as well as atypical varieties of the diphtheria bacillus, it follows that complete certainty must depend upon the isolation of the strain and examination of its fermentative properties and its toxigenicity. *C. hofmanni* fails to ferment glucose, and of course lacks the ability to form toxin.

An extensive literature exists on the possible transformation of *C. diphtheriae* to *C. hofmanni* (Hewlett, 1930; Gins, 1928). Because of the original belief, now generally discredited, that the latter represented a variant of the pathogenic species, the term "*pseudodiphtheria*" was originally associated with the Hofmann bacillus. At present it is generally accepted that the species is quite distinct, and the frequent association only one of chance.

### C. XEROSIS

Isolated originally from cases of xerosis conjunctivae, this organism was shortly

found to be present in many normal conjunctivae, and therefore is without pathologic significance. It is occasionally present in the throat, though much less commonly than the Hofmann bacillus. Morphologically it is probably indistinguishable from *C. diphtheriae*. Its growth on artificial media tends to be less vigorous than that of the diphtheria bacillus, and the colonies on tellurite plates are somewhat smaller than those of either the *mitis* or *gravis* strains, but larger than those of *intermedius* diphtheria. Here again, in case of doubt, one must resort to isolation, fermentation and virulence tests.

For convenience a condensed table of the more useful fermentations in this group is appended.

TABLE 22

	GLUCOSE	SUCROSE	STARCH
<i>C. diphtheriae, mitis</i> . . . . .	+	—	—
<i>C. diphtheriae, gravis</i> . . . . .	+	—	+
<i>C. diphtheriae, intermedius</i> . . . . .	+	—	—
<i>C. hofmanni</i> . . . . .	—	—	—
<i>C. xerosis</i> . . . . .	+	+	—

Other diphtheroids variable.

For a description of other varieties of corynebacteria from both human and animal sources, the reader should consult one of the larger handbooks of medical and veterinary bacteriology.

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## 10

# The Pneumococci

### INTRODUCTION

Gram-positive coccus, frequently of lancet shape, usually arranged in pairs or short chains, and possessing an easily demonstrable capsule. Pneumococcus is nonmotile and does not form spores. It is lysed by bile salts and other surface active agents. It is classified into types on the basis of immunologic and chemical differences in the highly polymerized polysaccharides which compose its capsule. It is a normal inhabitant of the upper respiratory tract of man and certain animals and causes infection primarily in the respiratory tract and adjacent structures, especially pneumonia, sinusitis, otitis, conjunctivitis and meningitis. (Figs. 1B and 2B.) (Synonyms: *Diplococcus pneumoniae*; *Streptococcus pneumoniae*.)

### HISTORY

Pneumococcus was first isolated and cultured in 1881 by Pasteur in France and independently in the same year by Sternberg in New York. In both instances saliva of persons who were not suffering from a respiratory disease was injected into rabbits from whose blood the organisms were subsequently isolated. Neither Pasteur nor Sternberg appreciated its relationship to disease. This was demonstrated by the independent studies of Frankel and of Weichselbaum between 1884 and 1886 who showed pneumococcus to be the most frequent cause of lobar pneumonia in man.

Classification of pneumococcus into types

began with the work of Neufeld and Handel in Germany in 1909 and 1910 with the observation that strains of pneumococci differ in their immunologic properties. Shortly afterward Dochez and Gillespie (1913) in New York subdivided pneumococci into three distinct types and a fourth heterogeneous group on the basis of agglutination reactions and protection tests in mice. Simultaneously, Lister in South Africa reported similar findings. The great significance in the observations of Dochez and Gillespie lies in the fact that two of the types described by them, Types I and II, are more commonly associated with pneumococcal pneumonia in adults than any of the other types and together are responsible for about one-half of all cases. Type III, the third type described by them, is more commonly carried in the normal human pharynx than any other single type of pneumococcus, and is likewise a frequent cause of pneumonia and other lesions. Subsequent study of the heterogeneous group IV of Dochez and Gillespie has resulted in the recognition of more than 75 distinct pneumococcal types.

The basis for the immunologic differentiation of pneumococci into types was demonstrated by Dochez and Avery (1917) to reside in the elaboration during growth of so-called specific soluble substances (SSS) which constitute the capsules of the micro



organisms. Heidelberger and Avery (1923) showed that the specific soluble substances are carbohydrate in nature, and to them and Goebel is due the greatest part of our knowledge of the immunologic and chemical properties of these capsular constituents. It has been convincingly shown that the production of the capsular polysaccharides is essential to the pathogenicity of pneumococcus, and that the antibodies which protect man or animals against infection are directed against this capsular material.

Many of the biologic properties of pneumococcus are similar to those of various species of streptococci, especially streptococci of the viridans group. It is for this reason that certain taxonomists have included pneumococcus as a species of streptococcus under the name *Streptococcus pneumoniae*, though agreement on this designation is not universal. The most important properties which distinguish pneumococcus from the green-producing streptococci are its predilection in causing pneumonia, its virulence for laboratory animals on primary isolation and its dissolution by bile salts and other surface active agents.

### MORPHOLOGY

In the sputum, pus and the lungs of patients with pneumonia, pneumococcus appears singly, in pairs or in short chains of ovoid or lanceolate cocci. When in pairs the adjacent ends of the cocci are generally rounded with the distal ends pointed. The appearance in artificial culture medium is similar except that short chains of cocci are more commonly seen, especially in young cultures. During the active phases of growth pneumococcus is Gram positive, but as the culture begins to age, Gram-negative forms appear which retain their morphology otherwise. On continued incubation, the Gram-positive forms gradually disappear and eventually are replaced entirely by Gram-negative cocci. This is followed by

further autolytic changes so that finally no formed elements but only Gram-negative debris can be seen.

The process of autolysis can be greatly enhanced by surface-active compounds. Upon the addition of whole bile or bile salts, such as sodium desoxycholate or taurocholate, for example, pneumococcus autolyzes with great rapidity, clearing of a turbid suspension of organisms occurring within a matter of minutes. The phenomenon of bile solubility is due to activation of the autolytic enzymes of pneumococcus, and if the enzymes are first inactivated by heating the suspension of cocci at 65° C. for 30 minutes, autolysis no longer takes place either spontaneously or when bile is added. The mechanism of activation of the autolytic enzyme system by surface-active substances has not been explained, though it seems not unlikely that activation results from alteration or removal from the cells of a normal inhibitor of autolysis.

On the surface of solid media such as fresh peptone-infusion agar plates to which blood has been added, young cultures of the virulent organisms form smooth, glistening, unpigmented, dome-shaped colonies which are circular in outline and in general vary from 0.5 to 1.5 mm. in diameter. Colonies formed by Type III pneumococcus are larger and more mucoid than those produced by other types and commonly attain a diameter of from 2 to 3 mm. on blood agar. The greater size and more mucoid consistency of the Type III colonies would appear to be due to the larger amount of capsular polysaccharide (SSS) synthesized by this type. As the cultures on blood agar age, autolytic changes appear, usually within a period of 24 hours. The centers of the colonies collapse, often leaving a small central papilla with a depressed area intervening between it and the raised outer margin of the colony.

Surrounding the colonies on blood agar and becoming more apparent with continued incubation at 37° C., there is a zone of

alpha hemolysis showing the characteristic greenish-brown color.

In fluid media pneumococcus grows diffusely and tends to sediment only when the pH has fallen because of acid production. This occurs in media containing relatively large amounts of glucose or other fermentable carbohydrates.

## NUTRITION

In the older literature pneumococcus is frequently characterized as a "fastidious" micro-organism, indicating difficulties in cultivation and maintenance in the viable state. These difficulties were probably multiple in nature and due not only to partial deficiencies in essential nutrients but also to improper oxidation-reduction potential of the medium.

The importance of the oxidation-reduction potential has been clarified especially through the studies of Dubos (1929). When peptone-meat infusion broth is exposed to the air, the medium becomes oxidized. Under such circumstances large inocula of pneumococcus must be used to obtain growth. The large inoculum is able through its metabolic activity to lower the redox potential sufficiently to permit growth, whereas with a small inoculum this may not occur. When the medium is reduced, on the other hand, whether by placing it under reduced pressure, by heating to drive off dissolved oxygen, or better, by addition of a reducing agent such as cysteine or thioglycollic acid, growth can be initiated from a very small inoculum. In practice, media satisfactory for pneumococcal growth can be prepared readily from fresh meat infusion with the addition of any good brand of peptone. The pH of the medium should be between 7.2 and 7.4 after sterilization, which should be accomplished with a minimum of heating. Media made with a fresh meat infusion base are in general much more satisfactory than the dehydrated media which are commercially available.

The optimum pH for growth of pneumococcus is stated to be 7.8. Excellent growth can be obtained over the range of pH 7.2 to 7.8, but from the practical viewpoint it is probably desirable to use media of pH 7.2 to 7.4 because there is less chance of deleterious alterations occurring on sterilization by heat at lower pH values.

All of the nutritional factors required for growth of pneumococcus have not been determined as yet. However, a partially defined medium has been prepared by Adams and Roe (1945) which supports the growth of many, but not all strains. This medium is basically that designed by Bernheimer et al. (1939) for the cultivation of Group A streptococci, with the addition of asparagine and choline which have been shown to be essential for growth of most pneumococcal strains tested. In addition to an acid hydrolysate of casein supplemented by *l*-cystine and *l*-tryptophane, the medium contains the following accessory growth factors: biotin, nicotinic acid, pantothenic acid, choline, pyridoxine, thiamine, riboflavin, adenine and uracil. Of these, the first four are known to be essential. In common with Group A streptococci, pneumococcus has been found to require large amounts of glutamine. Immediately before inoculation, sodium bicarbonate is added to provide CO<sub>2</sub> which is essential for growth, and the redox potential is lowered by addition of a reducing agent such as thioglycollic acid. Glucose is used as carbon source since pneumococcus derives almost all of its energy requirements from anaerobic glycolysis (fermentation). Table 23, which is taken from the paper by Adams and Roe, lists the components of the partially defined medium and the method of preparation. Further work is required to find out if all of the accessory growth factors listed are essential for pneumococcal growth, and to determine the amino acid requirements.

Defined media such as that described are especially useful for chemical investigations of bacteria, for example, the preparation of



## THE PNEUMOCOCCI

TABLE 23. COMPOSITION AND METHOD OF PREPARATION OF A PARTIALLY DEFINED MEDIUM FOR PNEUMOCOCCUS \*

*Basal Medium—for one liter of medium*

Acid hydrolysate of casein.....	200	ml. of 10% solution
<i>L</i> -Cystine.....	150	mg.
<i>L</i> -Tryptophane.....	20	mg.
KCl.....	3	Gm.
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O.....	7.5	Gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5	Gm.
Distilled water to make.....	900	ml.

Adjust pH to 7.5, heat to boiling, filter, and tube in 9 ml. amounts or appropriate multiple. Autoclave.

*Solution 1—vitamin mixture for 12.5 liters*

Biotin.....	0.015	mg.
Nicotinic acid.....	15.0	mg.
Pyridoxine.....	15.0	mg.
Calcium pantothenate.....	60.0	mg.
Thiamine.....	15.0	mg.
Riboflavin.....	7.0	mg.
Adenine sulfate.....	150.0	mg.
Uracil.....	150.0	mg.

Dissolve in 100 ml. of distilled water and sterilize by filtration. Store in refrigerator.

*Solution 2—salt mixture for 50 liters*

FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	50	mg.
CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	50	mg.
ZnSO <sub>4</sub> ·7H <sub>2</sub> O.....	50	mg.
MnCl <sub>2</sub> ·4H <sub>2</sub> O.....	20	mg.
HCl concentrated.....	1	ml.

Dissolve in 100 ml. of distilled water and sterilize by boiling.

*Addition mixture per liter of medium*

Vitamin mixture (solution 1).....	8.0	ml.
Salt mixture (solution 2).....	2.0	ml.
Glucose (20% solution).....	10.0	ml.
Glutamine.....	200	mg.
Asparagine.....	100	mg.
Choline.....	10	mg.
CaCl <sub>2</sub> ·2H <sub>2</sub> O.....	10	mg.
Distilled water to make.....	50	ml.

Sterilize by filtration and store in refrigerator. Add 0.5 ml. to each 9 ml. of basal medium. This addition mixture should not be kept longer than a few weeks as the glutamine is unstable. Solutions 1 and 2 appear to keep indefinitely.

*Bicarbonate—thioglycollate mixture*

Thioglycollic acid.....	10%
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Add 1 ml. of thioglycollic acid to 9 ml. of sterile distilled water, mix well and heat in boiling water bath for 10 minutes.

Bicarbonate. Weigh 200 mg. samples of sodium bicarbonate into test tubes and autoclave

Add 10 ml. of sterile distilled water to a test tube containing bicarbonate and dissolve the latter. Then add 0.2 ml. of 10 per cent thioglycollic acid and mix well immediately. Add 0.5 ml. of the mixture to each 9.5 ml. of medium. This bicarbonate-thioglycollate mixture is unstable and must be made up and added to the medium just prior to inoculation.

\* From Adams, M. H., and Roe, A. S., 1945, A partially defined medium for cultivation of pneumococcus. *Journal of Bacteriology*, 49, 401.

SSS, because all of the constituents are dialyzable and can be eliminated by this means. Moreover, for testing sulfonamide sensitivity of strains of pneumococcus, a medium of this type is a necessity because it is free from p-aminobenzoic acid and other sulfonamide inhibitors which interfere with sensitivity tests.

For routine cultivation of pneumococcus it is preferable to use complex media such as peptone-fresh meat infusion broth to which blood has been added. Growth of all strains can be obtained, autolysis is less rapid than in the defined medium described, and cultures can be stored in the icebox for prolonged periods of time. The presence of blood is particularly important for storing cultures. Presumably this is because of the catalase present in the red cells which destroys hydrogen peroxide produced by pneumococcus. Pneumococcus contains neither catalase nor peroxidase and in consequence  $H_2O_2$  accumulates in its environment if air is present, in amount probably great enough to affect viability of the organisms. In any case, although blood does not improve the growth of pneumococcus when added to a good medium, its presence favors preservation of viability of the organisms on storage.

### PHYSIOLOGY

As noted above, pneumococcus derives most of its energy requirements from the fermentation of glucose. Practically all of the glucose metabolized can be accounted for by the lactic acid which accumulates in the medium during growth. The metabolism of pneumococcus is thus essentially anaerobic. It can grow, however, in the presence or absence of oxygen and hence has been classified as aerobic or facultatively anaerobic. Growth is somewhat better under aerobic conditions even though pneumococcus does not possess the cytochrome system or catalase. Better growth under aerobic conditions is apparently due

to the capacity to take up oxygen through a flavin-containing enzyme system. Under these conditions  $H_2O_2$  is produced by the autoxidation of reduced flavoprotein. As previously noted,  $H_2O_2$  accumulates in the medium because pneumococcus possesses neither catalase nor peroxidase.

One of the most important factors limiting the growth of pneumococcus is the lowering of the pH of the medium due mainly to the accumulation of lactic acid, although with some strains significant amounts of formic and acetic acid may be produced (Friedemann, 1938). Massive growth can be obtained, however, by supplying a large amount of glucose and neutralizing the lactic acid with NaOH as it is formed.

The optimum temperature for growth is in the neighborhood of 37° C. Pneumococcus is rapidly killed when heated at 55° C. or above, but it should be emphasized that no sharp thermal death point can be defined since more prolonged heating at lower temperatures will also cause sterilization of a culture.

### IDENTIFICATION

Members of the heterogeneous group of organisms collectively described under the term "viridans streptococci" are the only bacteria which are commonly confused with pneumococcus. A single test, however, may be used to separate them—that is, whether they are bile soluble or not. All, or practically all, strains of pneumococcus are bile soluble, whereas the viridans streptococci are insoluble. This test is extremely reliable, provided the conditions are proper and appropriate controls used. In general, it is advisable to remove the living organisms by centrifugation from the medium in which they have grown, especially if it contains blood or other protein materials. The organisms should be suspended in isotonic saline, preferably adjusted so that the pH is about neutral, although the range between pH 6.5 and 8.0 is satisfactory. Un-



der these circumstances pneumococci go into solution with great rapidity either at room temperatures or 37° C. upon the addition of an equal volume of 10 per cent bile. Dissolution is faster at 37° C., and if the suspension has not cleared within 30 minutes at this temperature, it is most unlikely that the micro-organism in question is pneumococcus. As a control, the living organisms prepared as described above may be used, with an equal volume of saline substituted for bile. Alternatively, one may use as control a cell suspension of the organism to be tested, previously heated at 65° for 30 minutes, to which bile is then added, or else a suspension prepared from a known culture of living pneumococcal cells to which bile is added.

The fermentation of inulin is generally given as a differential test for distinguishing pneumococcus from streptococci. While it is true that inulin fermentation is a characteristic property of pneumococcus, it is not a reliable test when used by itself since certain streptococci, especially those of the *Salivarius* group, share this capacity.

Another property which is of great use in identifying pneumococcus is its virulence for mice on experimental inoculation. Streptococci of the viridans group are avirulent for mice, whereas most strains of pneumococci are highly virulent even on primary isolation. There are important exceptions, however, since certain pneumococcal types, for example, Type XIV, are not mouse virulent.

### VARIATION

On cultivating encapsulated pneumococci in nutrient broth to which has been added serum containing antibodies to the capsular polysaccharide, after a few transfers most of the pneumococci will be found to be devoid of capsules (Stryker, 1916). For example, when pneumococcus Type I is grown in the presence of Type I antipneumococcal serum, encapsulated Type I pneumococci disappear and are replaced by nonencapsu-

lated forms. Type II pneumococcus grown in the presence of Type II antibody behaves in the same way. With loss of the capsule, the organisms lose type specificity and virtually all of their pathogenicity.

On the surface of an agar medium encapsulated pneumococci form characteristic smooth, glistening colonies. The pneumococci composing the smooth colonies are referred to as *smooth* or S organisms. Upon loss of the capsule, such as occurs on cultivation in homologous antiserum, the colonies formed on agar are no longer smooth but have a finely roughened surface and for this reason the organisms composing them are spoken of as *rough* or R.

When R pneumococci are subcultured repeatedly in broth containing anti-R serum, in most instances the R pneumococci disappear and are replaced by S pneumococci of the same serologic type as that from which the R strain was originally derived. Virulence is restored at the same time.

It has been customary to speak of anti-S serum as "causing" reversion from R to S, thereby inferring that anti-S serum in some way depresses capsular synthesis and anti-R serum stimulates it. This is almost certainly an incorrect view. Any pneumococcal culture, although superficially homogeneous, undoubtedly contains a large number of variants, and in all probability S cultures throw off R mutants at a fairly constant rate. It has been shown experimentally that if a mixture of S and R cells is inoculated into broth containing anti-S serum, R cells have an enormous selective advantage over S and conversely when such a mixture is grown in anti-R serum, S organisms have a selective advantage over R forms (MacLeod and Krauss, 1947). It is most probable, therefore, that anti-S serum does not "convert" S cells to the R form, but rather that the R mutants present in the S culture are selected specifically by this means. The occasional S forms in an R culture are selected similarly when cultivation is carried

on in an anti-R serum, R organisms tending to disappear.

The enhancement of virulence which occurs upon repeated animal passage is in all likelihood a similar process of selection. The less virulent forms are destroyed by the host, the more virulent are able to survive and thus are specifically selected. Selection of drug-resistant variants or mutants by cultivating the organisms in increasing concentrations of a particular drug may also be cited as another example of the same process.

A phenomenon which appears at first sight to be related to the selection of variants capable of surviving in a particular environment is that of pneumococcal type transformation. As shown originally by Griffith (1928), pneumococcus of one specific type can be transformed to another specific type by way of the R variant. In his original experiments Griffith obtained R cells from a culture of pneumococcus Type II by cultivation in Type II antiserum. The living R cells derived from Type II were then subcutaneously inoculated into mice along with large numbers of heat-killed Type III S cells. Upon death of the mice, cultures of the heart blood showed the presence of living Type III S cells. In other words, he was able to transform cells which were derived from a culture that originally synthesized the Type II capsular polysaccharide into cells producing the Type III capsule. Subsequently, Dawson and Sia (1931) accomplished the same result in vitro using heat-killed S cells as the transforming agent, and Alloway (1932) was able to effect transformation using filtered, cell-free extracts of S cells. The more recent studies of Avery, MacLeod and McCarty (1944) indicate that the active transforming principle present in S cells is a highly polymerized form of desoxyribonucleic acid. It should be emphasized that the specific capsular polysaccharides (SSS) are not the active agents in bringing about pneumococcal type transformation.

Type transformation differs greatly from the process of selection of mutants under particular environmental conditions in that the changes effected through transformation can be specifically directed. This fact makes the transformation reaction an extremely important one for the study of genetic mechanisms.

## ANTIGENIC STRUCTURE

Pneumococcus can be divided into 75 or more types on the basis of differences in the capsules which surround the cells. The capsules are composed of highly polymerized polysaccharides which are immunologically distinct for each type. Extensive studies carried out especially by Heidelberger, Goebel and Avery have provided a chemical basis for the immunologic specificity of the capsular polysaccharides of a number of types. Table 24 shows certain of the chemical properties of polysaccharides of Types I, II, III and VIII.

The isolated, purified capsular polysaccharides of pneumococci are antigenic for some species such as man and mouse, but not for the rabbit, although the serum of rabbits immunized by the injection of intact pneumococcal cells contains antibodies which react specifically with the isolated capsular polysaccharides of the same type. That is to say, the polysaccharides are haptens for the rabbit but antigens for man and mouse. Antibodies directed against the capsular polysaccharides protect against infection. The evidence for this is discussed under "Pathogenesis."

The somatic portion of the cells is antigenically similar for all types despite the immunologic specificity of the capsular polysaccharides surrounding it. Moreover, antiserum to the somatic portion of the cell shows cross reactions with extracts prepared from certain streptococci. A detailed immunologic analysis has not been made of the various protein constituents of pneumococcus. Furthermore, R pneumococci derived



TABLE 24. PROPERTIES OF THE SPECIFIC CAPSULAR POLYSACCHARIDES (SSS) OF FOUR PNEUMOCOCCAL TYPES \*

TYPE	OPTICAL ROTA- TION	ACID EQUIV- ALENT	N	REDUCING POWER ON HYDROLYSIS AS GLUCOSE	CONSTITUENTS IDENTIFIED	COMMENT
I	+280°	650	<i>per cent</i> 4.6 (Amino N 2.0)	<i>per cent</i> 30	Galacturonic acid Amino sugar Acetyl, one group per equivalent	Basic unit a trisaccharide con- taining 2 uronic acid mole- cules and an amino sugar
II	+55°	950	0.2	95	Glucose Aldobionic acid (Glucose and glucuronic acid)	Cross reacts with antiserum to K. friedländerii Type B
III	-35°	340	0.1	85	Aldobionic acid (Glucose and glucuronic acid)	Cross reactions between the polysaccharides of Types III and VIII are marked espe- cially with antiserum pre- pared by immunizing horses.
VIII	+123°	720	0.2	87	Aldobionic acid (Glucose and glucuronic acid) Glucose, 2 molecules per molecule of aldobionic acid	Cross reactions much less with rabbit antiserum

The small amount of N found in Types III and VIII polysaccharides is most probably due to impurities in the preparations.

\* These data are from the summary of Heidelberger, M., *Revue d'immunologie*, 1938, 4, 293; and that of Mar-  
rack, J. R., Medical Research Council Special Report Series No. 230; London, His Majesty's Stationery Office,  
1938.

from one type are agglutinated to almost the same titer by antisera prepared against R pneumococci derived from heterologous types as by homologous antisera.

Like  $\beta$ -hemolytic streptococci, the somatic portion of pneumococcus contains a C or cellular carbohydrate described by Tillett, Goebel and Avery (1930) which appears to be immunologically as characteristic of pneumococcus as a species as are the C carbohydrates for the Lancefield groups of streptococci. Unlike  $\beta$ -hemolytic streptococci, however, the C carbohydrate of pneumococcus has not been used as a means of classifying pneumococci from the systematic point of view, though there is good reason to believe that it can be used in this way. The studies of Goebel and Adams

(1943) have shown that the C carbohydrate forms a portion of the Forsmann (heterophile) antigen of pneumococcus.

Antibodies to the whole somatic portion of pneumococcal cells or any fractions of it so far studied show only modest protective power for experimental animals in contrast with the great protection given by antibodies to the capsular polysaccharides. Tillett (1928) demonstrated that rabbits immunized with heat-killed R pneumococci develop resistance to infection with virulent, encapsulated pneumococci of Types I, II and III. Subsequently Dubos (1938) extracted a soluble antigen from pneumococcal cells which caused the production in rabbits of antibodies capable of protecting mice against infection with both homolo-

gous and heterologous types. The nature of the antigen responsible for the broad immunity studied by Tillett and by Dubos is unknown, though there is evidence that the C polysaccharide may be involved (Enders, Wu and Shaffer, 1936). It is worth emphasizing, however, that non-type-specific resistance to infection obtained by the methods described above is slight when compared with the high degree of immunity afforded by type-specific antibodies, and probably plays a minor part in protection against pneumococcal infection.

### DISTRIBUTION

*Pneumococcus* has a world-wide distribution. It is a normal inhabitant of the nasopharynx of man under all climatic conditions. In all parts of the world Types I and II are most important in the causation of human disease, though in the normal subject types other than these are more commonly carried. Among other animal species, guinea pigs and monkeys are the only ones that are known to harbor pneumococci commonly. These species have little or no importance as reservoirs of pneumococci as far as human disease is concerned. Epizootics of pneumococcal pneumonia occur among monkeys in captivity, however, and in the guinea pig, pneumococcus Type XIX causes one of the most frequent and fatal epizootic diseases of this species. None of the other types is of importance in causing disease in guinea pigs though they may become carriers if other types are experimentally implanted in the nasopharynx.

### PATHOGENICITY AND HOST RANGE

Infections in man can be caused by any of the more than 75 serologic types of pneumococcus. Of this large number of types, however, a relatively small number accounts for most human disease. Types I and II together cause approximately one-half of all the cases of lobar pneumonia in adults in

all parts of the world (Heffron, 1939), with eight types causing about 75 to 80 per cent of the total number of infections. Table 25 which is taken from the summaries of Heffron shows the incidence of lobar pneumonia caused by various types in three large cities in northeastern United States.

TABLE 25. DISTRIBUTION OF PNEUMOCOCCAL TYPES IN ADULTS WITH LOBAR PNEUMONIA

PNEUMOCOCCAL TYPE	NUMBER OF CASES	PER CENT OF TOTAL
I.....	1,063	28.6
II.....	425	11.4
III.....	500	13.5
IV.....	131	3.5
V.....	298	8.0
VI.....	66	1.8
VII.....	240	6.5
VIII.....	287	7.7
Total I-VIII.....	3,010	81.0
All other types....	703	19.0
	3,713	100.0

It should be noted that the figures given in Table 25 are for cases of pneumonia having anatomically a lobar distribution. Pneumococci also are the most frequent causative agents among bacteria of bronchopneumonia as well, but the accuracy of the figures for this anatomical variety is not so great as for lobar pneumonia.

In children below the age of twelve the distribution of pneumococcal types in lobar pneumonia differs from that in adults, with Types XIV, I, VI, V, VII and XIX in that order, causing more than half the cases (Heffron). It is of interest that the capsular polysaccharide of Type XIV is immunologically related to blood group A substance (Beeson and Goebel, 1939).

Among laboratory animals, the mouse is the most susceptible species although rats and rabbits are also highly susceptible upon experimental inoculation. The virulence of most types for mice can be enhanced quickly



by repeated passage, presumably due to the selection by this means of the most virulent variants from a mixed population of cells of variable virulence. With many types, as few as 1 to 5 cocci inoculated intraperitoneally in mice will cause death within 48 hours. An important exception is Type XIV, however, which is avirulent for mice despite the fact that it is one of the most frequent causes of lobar pneumonia in children. It is probable that if systematic study were made, other types which are uniformly avirulent for mice would also be found.

Similar observations have been made in the rabbit with pneumococcus Type III which, although highly virulent for man and mouse, is almost avirulent for the rabbit. An occasional strain of Type III has been described, however, which possesses pathogenic properties for rabbits. A satisfactory explanation for the difference in virulence of these strains of pneumococcus Type III has not been found.

Both dogs and monkeys have been used extensively for the study of pneumococcal lobar pneumonia produced by experimental inoculation by the intratracheal route and under appropriate conditions both are highly susceptible. Felines are relatively insusceptible to experimental infection, and birds are especially resistant.

It is apparent from the observations on pathogenicity described above that the data obtained from studies of one animal species cannot be transferred to another. Virulence, in the case of pneumococcus should be defined only for the species in which it has been measured, and one cannot infer, for example, that because a strain or type is virulent for the mouse it is comparably virulent for man or vice versa.

#### FACTORS INVOLVED IN PATHOGENICITY OF PNEUMOCOCCUS

Pneumococcus is perhaps the best illustration of a bacterial species that produces disease apparently solely through invasive

properties, in other words, because of the capacity to invade and multiply in living tissues without evidence that soluble toxins, in the usual sense, play a part. *Cl. botulinum* at the other end of the scale, exerts its pathogenicity entirely through a potent exotoxin which is produced outside of the body and causes disease upon absorption through the gut. *Cl. botulinum* has no invasive capacity whatsoever. Between these two extremes of "purely" invasive and "purely" toxigenic pathogens are many species that possess both capacities in varying degrees.

The search for a toxin produced by pneumococcus which can account for its disease-producing capacity has been unsuccessful. It is true that two "toxic" principles have been identified, but since neither of them is liberated in significant amount except upon autolysis of the bacterial cells it is doubtful that either plays a significant part in pneumococcal pathogenicity.

Pneumolysin, an oxygen-labile or O hemolysin, is liberated from pneumococcus especially on autolysis (Cole, 1914). It is related serologically to the O hemolysins produced by hemolytic streptococci, *Cl. tetani* and *Cl. welchii*, as shown by Todd (1934). It should be noted, however, that hemolysis is never a feature of even overwhelming pneumococcal infection, and also that in the case of the three toxigenic species noted above which produce similar O hemolysins, it has not been proved that these substances play a significant part in disease caused by the respective species.

Under conditions of autolysis there is also liberated from pneumococcus a "purpura-producing principle" whose effect can be demonstrated both in the skin and internal organs of experimental animals injected with sterile pneumococcal autolysates. Once again, however, purpura or other hemorrhagic incidents are not seen except in the rarest instances in pneumococcal infection whether in man or other animals, so that the purpura-producing principle would ap-

pear to have little significance in the pathogenicity of pneumococci.

As far as present information goes, it appears that pneumococci produce disease and death solely through their capacity to multiply in the tissues. It is possible, however, that a toxin is produced in the tissues which differs in nature from any previously studied, and that its demonstration requires new methods. Such a toxin might be a normal metabolic end-product, for example, which, because of the very rapid multiplication of the bacterial cells, accumulates locally and damages tissues through upset of their normal metabolic processes.

Another possible mechanism whereby such a rapidly growing pathogen might produce tissue damage is through the production of local or general acute deficiencies in one or more essential metabolites, whether vitamins, amino acids, purines or constituents of other cell components. The demands of bacterial cells having a generation time of from 15 to 20 minutes on available supplies of essential metabolites in the animal body must be great indeed, and conceivably the micro-organisms might compete for limited supplies of such materials on a more favorable basis than the cells of the host, with a resulting acute deficiency for the host cells. Other means by which a primarily invasive organism such as pneumococcus causes tissue damage might be suggested but would serve only to emphasize our state of ignorance.

Whatever may be the ultimate means by which pneumococcus produces damage to tissues, a fact of great significance is that most of the pathogenic power of this species is exerted through possession of a nontoxic surface component—the pneumococcal capsule. All virulent strains of pneumococcus possess a capsule which forms a protective mantle about the cells. The sole function of the pneumococcal capsule appears to be to delay or prevent ingestion of pneumococci by the phagocytic cells of the body. The free, isolated polysaccharides which com-

pose the capsules in the different types are nontoxic upon injection in even very large doses. Evidence of the role of the capsule in pathogenicity has been obtained in a variety of ways, a number of which will be described.

Upon immunization of experimental animals by injecting them with killed, encapsulated pneumococci, their serum contains antibodies which will specifically protect other animals infected with living, virulent pneumococci of the same type as that used for immunization. For example, a serum prepared by immunizing rabbits with heat-killed pneumococcus Type I protects against experimental infection with living Type I pneumococci, but will not protect against infection with Type II pneumococcus. In other words, the protective antibodies are type specific.

The capsular polysaccharides of several types of pneumococcus have been prepared in relatively pure form as previously noted. It has been shown that the protective capacity of a serum depends upon the amount of antibody present which is capable of reacting with the capsular polysaccharides. By the addition of an appropriate amount of purified specific capsular polysaccharide to a highly protective serum, all of the anticapsular antibodies can be precipitated and removed. A serum absorbed in this way loses its protective power. Anticapsular antibodies can also be absorbed from a serum by permitting it to react with whole encapsulated pneumococcal cells, and here again the protective power is removed at the same time. However, one cannot conclude from the results of absorption with whole bacterial cells that it is the anticapsular antibodies that are of importance in protecting against infection, since other antibodies, reacting with other components of the pneumococcal cell, may be removed simultaneously. Absorption of serum with purified capsular polysaccharides, on the other hand, shows unequivocally that it is the antibody to this material that is of importance in



protection against infection and at the same time indicates clearly the importance of the capsule in the pathogenicity of the organism.

Even more direct evidence for the importance of the capsule in pneumococcal pathogenicity is found in the fact that on immunizing with highly purified capsular polysaccharides, man and mouse produce antibodies which give a precipitin reaction with the homologous polysaccharides, agglutinate encapsulated pneumococci and protect specifically against infection by the homologous type. On the other hand, immunization with R organisms results in production of serum of only slight protective power (Tillett, 1928). Anti-R sera do not agglutinate S organisms or give a precipitin reaction with the capsular polysaccharides.

The loss of the capsule in R organisms deprives the cells of most of their virulence, though not all. With fully virulent, encapsulated strains, a dilution of broth culture containing from 1 to 5 viable organisms ( $1 \times 10^{-8}$  cc. of culture) will kill mice following intraperitoneal injection. With R organisms selected from the same strain by growing in anti-S serum, 0.5 to 1.0 cc. of culture is necessary to cause death or approximately 100,000,000 to 200,000,000 living R organisms. Removal of the capsule, therefore, has decreased virulence roughly 100,000,000 ( $10^8$ )-fold.

The antiphagocytic property of the pneumococcal capsule can be easily demonstrated *in vitro* by mixing R and S pneumococci, respectively, with heparinized normal human blood. Phagocytosis of the R cells takes place with great rapidity, whereas the leukocytes have a limited capacity to phagocytose the S forms. If a small amount of type-specific antiserum is added, however, the S cells are rapidly taken up by the leukocytes. The combination of specific antibody with the capsular polysaccharide removes its antiphagocytic property. The antiphagocytic effect of the capsule is in all likelihood not due to any toxic action on

the leukocytes, but would appear rather to be a mechanical one. The detailed studies of Wood and his co-workers (1946) on this topic indicate that when phagocytosis experiments are carried out on smooth surfaces such as glass or wax, encapsulated pneumococci are pushed about by the leukocytes, which seem to be unable to enclose the organisms within the pseudopodia because the pneumococci slide away over the smooth surface. On a roughened surface such as blotting paper or filter paper, on the other hand, the leukocytes take up encapsulated pneumococci fairly readily even in the absence of specific antibody because the bacteria can be pinned against obstructions on the roughened surface and then enclosed in the pseudopodic extensions. Intracellular digestion ensues.

If antibody is added to the mixture of encapsulated pneumococci and phagocytes, the nature of the surface on which the experiment is carried out makes little difference. The combination of antibody with the capsule appears to make the pneumococci sticky since now they adhere to the leukocytes and are very quickly ingested (Wood et al., 1946).

From the foregoing data, it should be apparent that the presence of type-specific antibody is not absolutely necessary for phagocytosis of encapsulated pneumococci to occur. In the absence of antibody the rate of phagocytosis is relatively slow but it is enormously enhanced when antibody is added.

The studies of Avery and Dubos (1931) on an enzyme which specifically digests the capsular polysaccharide of pneumococcus Type III both when the polysaccharide is in the isolated, purified state and when it is present on the surface of the living, virulent cells, provide further evidence for the importance of the capsule in pathogenicity of pneumococci.

From the soil of a cranberry bog an aerobic, sporulating bacillus was isolated, which when grown in a medium containing

the polysaccharide of pneumococcus Type III as the sole carbon source, elaborates a specific adaptive enzyme having the capacity to hydrolyze the capsular polysaccharide of pneumococcus Type III, but none of the other pneumococcal polysaccharides. In the test tube, the Type III enzyme digests and removes the capsule from either dead or living Type III pneumococci, and moreover, when injected into mice, rabbits or monkeys infected experimentally with pneumococcus Type III, as long as 18 hours after infection, the enzyme prevents the death of the animal. The sole action of the SIII enzyme is to remove the pneumococcal capsule by digesting it with the result that the decapsulated bacteria become highly susceptible to phagocytosis. It should be emphasized that digestion of the Type III capsule from the living organisms *in vitro* in no way affects their viability, though as long as active enzyme is present, the capsular material is digested as rapidly as it is produced. However, when transferred to fresh medium lacking the SIII enzyme, the pneumococci again produce their capsules normally.

A considerable body of evidence has been accumulated, therefore, which emphasizes the importance of the pneumococcal capsule in pathogenicity. The part played by somatic factors, on the other hand, has received little attention though there is evidence of their significance. One of the most clear-cut illustrations of the influence of somatic factors is in the case of two well-known laboratory strains of pneumococcus Type III, known as A66 and SV-III. Strain SV-III is highly virulent for rabbits, whereas A66 has very little virulence even upon repeated animal passage. The capsular polysaccharide appears to be identical in both strains. Moreover, if the capsule is switched from A66 to SV-III and vice versa, by means of the transformation reaction, no alteration in virulence occurs (Shaffer, Enders and Wu, 1936). In other words, the difference in virulence of these strains depends

on the somatic portion of the cells and not on the capsule.

Pneumococcus may be avirulent for a species because of the nature of the capsule. Type XIV, for example, is avirulent for the mouse on this basis. R pneumococci derived from avirulent Type XIV pneumococcus become highly mouse virulent when transformed to pneumococcus Type II and similarly when virulent Type II pneumococci are transformed to Type XIV by way of the R variant, the newly constituted Type XIV strain is as avirulent for the mouse as naturally occurring Type XIV pneumococci (MacLeod and McCarty, 1942).

### PATHOGENESIS OF PNEUMOCOCCAL INFECTION

Pneumococcal pneumonia is rarely a primary infection but usually follows damage to the respiratory tract caused by some unrelated agent whether viral or chemical. In most instances, pneumonia is preceded by an upper respiratory infection such as common cold or influenza. Pneumococcal pneumonia may be classed, therefore, as a complex infection. This relationship to viral infections probably explains why pneumococcal pneumonia is commonest during the cold months of the year in northern latitudes, since common colds and influenza occur with greatest frequency at these times. It is unlikely that the virulence of pneumococci for man varies with the seasons.

It is apparent from the relation of pneumococcal pneumonia to viral infection that the normal respiratory mucosa must possess great natural resistance to pneumococcus, especially when it is recalled that between 40 and 70 per cent of all normal humans are carriers of pneumococci, many types of which are potentially virulent. The factors normally involved in protection of the respiratory mucous membrane have not been defined, and we are also ignorant of the nature of the predisposing injury caused by viruses or chemical irritants such as gases,



though it seems likely that actual destruction of the superficial cell layers may be important in permitting invasion of underlying tissues. Recent evidence indicates that the possession of type-specific antibody not only protects man against infection under natural conditions but also renders him less likely to become a carrier of pneumococci of homologous type (MacLeod et al, 1945). However, it is reasonably certain that, in the main, normal resistance to pneumococcal pneumonia does not depend upon the possession of antibodies reactive with the many types with which one comes in contact, but rather upon other factors including the accident of whether one happens to be a carrier of a highly pathogenic type such as Type I or II.

In the healthy adult, pneumococcal pneumonia characteristically involves one or more lobes or a discrete portion of them, leaving the remaining bronchopulmonary system relatively uninvolved. In infants, in young children and in the aged, the lesions more commonly follow a bronchial distribution without the localized character of lobar pneumonia.

Experimental pneumococcal pneumonia has been studied in the monkey by Blake and Cecil (1920) and in the dog by Robertson and others (see Terrell et al., 1933). In both species pneumonia can be produced successfully by administering a narcotic such as morphine to depress respiration and the cough reflex, and then introducing virulent pneumococci into the trachea or directly into a portion of a pulmonary lobe by means of a fine catheter. In the dog an important factor is the production of atelectasis in the portion of the lobe infected, and it seems likely that partial atelectasis may be important also in the etiology of lobar pneumonia in man. The increased bronchial secretions and edema occurring during the common cold or influenza may play a significant part through plugging of bronchioles and the production of areas of atelectasis, and in addition, the tendency

for secretions to pool in the most dependent portions may explain in part why the lower pulmonary lobes are most frequently involved.

### PATHOLOGIC AND CLINICAL PICTURE

The lesion in the lung consists essentially of marked edema of the alveolar walls with an outpouring into the alveoli of fibrinous exudate containing large numbers of red blood cells and polymorphonuclear leukocytes. The affected lung, therefore, becomes consolidated. The overlying pleura is involved early, with a serous pleural effusion as a common incident. Pneumococci in large numbers can be seen throughout the inflamed area, but, despite their presence both in the alveoli and the alveolar septae, necrosis does not take place. The absence of necrosis of the alveolar septae explains in all probability why upon recovery from the disease the lung is able to return to its original state. This is also indirect evidence that pneumococcus does not produce a potent exotoxic principle.

Sputum, which is usually produced throughout the disease, is similar to the alveolar exudate. It is characteristically bloody or rusty, the red cells being intimately mixed with the other components. Large numbers of pneumococci are usually present and can be quickly identified by immunologic methods, especially the Neufeld "quellung" or capsular swelling reaction.

The onset of acute lobar pneumonia is characteristically sudden, often with the occurrence of a chill and sharp pleural pain. Recovery of untreated cases frequently occurs likewise with dramatic suddenness—the so-called pneumonic crisis which generally occurs within 5 or 10 days from the initial chill.

Pneumococci can be recovered from the blood in a variable proportion of cases, the incidence of bacteremia depending in good part on the frequency with which cultures

are made and the technic followed. In the large series of cases compiled by Heffron (1939) bacteremia occurred in 26 per cent. All observers agree that persistent and increasing bacteremia is a grave prognostic sign.

In the period before the introduction of chemotherapy, empyema was the commonest complication of lobar pneumonia, occurring in approximately 3 per cent of patients (Heffron).

The evidence indicates very strongly that spontaneous recovery from pneumococcal pneumonia is dependent upon the development of type specific antibody which can be demonstrated usually in the blood at about the time of crisis. It should be remembered, however, that the antibody demonstrable in the circulating blood represents the excess left over after combination with type specific polysaccharide present in the lung and other organs of the body. In other words, antibody is being formed before recovery is apparent, but appears in sufficient excess at that time so that it can be detected in the blood serum.

## LABORATORY DIAGNOSIS

The effective use of specific serum therapy for treatment of pneumococcal pneumonia necessitated rapid and accurate methods of etiologic diagnosis. Of the many methods described, the Neufeld "quellung" or capsular swelling reaction is the simplest and best. Neufeld observed in 1902 that when pneumococci of a particular type and homologous antiserum are mixed together, the capsules of the organisms become greatly swollen and in this state are clearly visible under the microscope. The swelling is due to combination of specific antibody with the capsular polysaccharide.

Sputum is emulsified by drawing it repeatedly into a syringe. It is then stained by Gram's method to determine whether organisms having the morphology of pneumococci are present. Loopfuls of emulsified

sputum are then mixed with a loopful of the various specific antipneumococcal sera, a loopful of methylene blue added to make the somatic portion of the cells more easily visible, and the preparation examined immediately under the oil immersion lens. In a positive reaction, the pneumococcal capsule has a greatly swollen appearance and

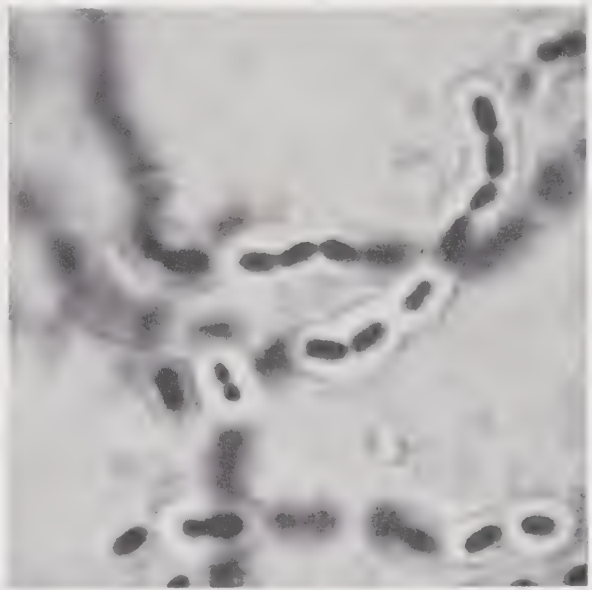


FIG. 6. *Pneumococcus* type III capsular swelling in the presence of type III antibodies, magnified  $\times 1,350$ . Capsular swelling with pneumococcus type III is more marked than with other types due to the greater production of SSS by type III. (Dr. G. W. Rake of the Squibb Institute for Medical Research.)

its border is sharply delimited from the surrounding medium. The Neufeld reaction can be used to identify pneumococci in sputum, in the exudates from experimentally infected animals and when grown in artificial culture media. Under the latter circumstances, very young cultures only should be examined, since in older cultures much of the capsular material diffuses away from the pneumococci into the surrounding medium, so that capsular swelling is much less striking.

Diagnosis can also be made by injecting mice intraperitoneally with sputum. In general, pneumococci are much more patho-



genic for the mouse than other organisms present in sputum or saliva and consequently come to predominate within a few hours in both the peritoneal exudate and blood of the mouse. Upon death of the mouse, which usually occurs from 16 to 48 hours following injection of sputum, some of the peritoneal exudate is removed, examined microscopically after Gram staining, and then typed by the Neufeld reaction. Pneumococci can usually be recovered in pure culture from the heart blood of the infected mouse. It is good practice to make cultures of the heart blood for confirmatory studies.

Pneumococci can be typed also by agglutination and precipitin reactions, but the simplicity and great accuracy of the Neufeld reaction make it a preferable technic.

Culture of the blood of patients with pneumonia is important from the diagnostic point of view but even more so as a guide in prognosis.

### SPECIFIC SERUM THERAPY

Pneumococcal pneumonia is one of the few infectious diseases for which effective specific therapy has been evolved. This was made possible mainly through the fundamental studies of Avery, Chickering, Cole and Dochez (1917) who first showed convincingly that the antiserum to be used must be type specific, that antibody must be given in adequate amounts and that it is most effective when administered early in the course of the disease. Applied originally to treatment of pneumonia caused by Types I and II, highly potent antisera later became available commercially for pneumonia caused by the majority of pneumococcal types (for summaries see Bullowa, 1937; and Lord and Heffron, 1938).

The earliest antisera were prepared by the immunization of horses with killed pneumococci and were administered intravenously in unconcentrated form. Subsequently, various methods were developed for refining and

concentrating the antibody globulins in the crude serum which greatly facilitated treatment. The introduction of rabbits for preparing antipneumococcal serum for therapeutic purposes was another advance since, in general, higher titers of antibody than in the horse could be obtained (Horsfall et al., 1937).

The dosage of type specific antipneumococcal serum can be controlled by determining whether the patient's blood and tissues contain an excess of antibody. Specimens of blood serum obtained at intervals after treatment can be tested for free antibody by means of agglutination reactions with suspensions of homologous pneumococci. Dosage of antiserum is adjusted so that an excess of antibody is constantly present in the blood. A more useful method, however, is the skin test with the purified specific capsular polysaccharides described by Francis (1933): 0.1 mg. of homologous specific capsular polysaccharide, dissolved in 0.1 cubic centimeters of saline, is injected intracutaneously. If circulating antibody is present, a wheal and erythema reaction appears at the site of polysaccharide injection within 15 or 30 minutes. The wheal and erythema are due to the combination locally of polysaccharide and homologous antibody. As early as possible in the course of the disease, antibody is administered intravenously in an amount sufficient to result in a positive polysaccharide skin test. In most instances, defervescence and the onset of recovery occur within a few hours after sufficient antibody has been given. At intervals of a few hours after serum therapy was first given, the polysaccharide skin test is repeated in order to make sure that antibody remains present in excess.

The ability to make a rapid etiologic diagnosis by means of the Neufeld reaction, the preparation of highly concentrated preparations of specific antibody and control of dosage by the polysaccharide skin test together made the serum treatment of pneumococcal pneumonia highly effective. On

the other hand, the specialized nature of the technics involved, the necessity for maintaining stocks of many type specific antisera, and the constant fear of anaphylactic reactions following intravenous administration of foreign protein, prevented the serum treatment of pneumococcal pneumonia from achieving general use.

### CHEMOTHERAPY

Pneumococci are highly susceptible to most of the sulfonamide derivatives in common use and these compounds can be used effectively in treatment. Sulfonamide-resistant mutants appear especially if the drugs are administered in suboptimal dosage for a prolonged period. The participation of specific immunity appears to be necessary for a successful outcome in sulfonamide therapy (MacLeod, 1939).

Pneumococcal infections can also be treated with penicillin with extremely good results. The development of penicillin-resistant mutants appears to occur in rare instances only.

It is instructive to compare the mechanism of action of specific serum therapy and chemotherapy in combatting pneumococcal infections. Spontaneous recovery from pneumococcal pneumonia is associated with the appearance of specific antibody in the blood over and above the amount required to combine with the capsules of the organisms and thus render them susceptible to phagocytosis. In the natural disease there is, therefore, a competition between the capacity of the pneumococci to grow and produce SSS and the ability of the infected person to form antibody to it. Treatment with specific antiserum tips the balance in favor of the host since sufficient can be given in a short time to combine with all the SSS present, both that which is on the surface of the micro-organisms and that which is free in the blood and tissues.

The sulfonamide drugs, which are essentially bacteriostatic, act by restraining the

growth of the organisms until sufficient antibody has been formed in the body to assure sensitization and phagocytosis of the pneumococci. As might be expected, the sulfonamide drugs and specific antipneumococcal serum exert a synergistic effect (MacLeod, 1939).

In the case of penicillin, which is essentially a bactericidal compound when used in full therapeutic dosage, dependence upon specific antibody is less than with sulfonamides, although there is some evidence that penicillin is less active against those pneumococcal types which are the weakest antigenically (MacLeod and Stone, 1945).

### EPIDEMIOLOGY OF PNEUMOCOCCAL PNEUMONIA

As noted above, pneumococcal pneumonia occurs almost always secondary to injury to the respiratory mucosa caused by an unrelated agent such as a virus infection or irritating gas. Furthermore, the virulence of the various pneumococcal types for man differs greatly. Types I and II have the most pronounced human virulence, since between them they cause about one-half of all the cases of lobar pneumonia in the adult. On the other hand, certain other types are encountered very rarely as the cause of pneumonia, and hence can be considered as of low virulence. It should be apparent, therefore, that the chances of developing pneumococcal pneumonia depend in great part upon whether the nonimmune individual is a carrier of one of the more highly pathogenic pneumococcal types at the time he comes down with a viral infection of the respiratory tract such as common cold or influenza. There is ample evidence (summarized by Heffron, 1939) that when pneumococcal pneumonia is epidemic in a community it is always associated with a high carrier incidence of the pneumococcal types causing disease. Significantly, most of the epidemics of pneumococcal pneumonia reported in the literature have been caused by



the same types that are responsible for most of the cases of endemic pneumonia: Types I, II, IV, V and VII. Under normal circumstances the incidence of carriers of the highly pathogenic types is relatively low. However, if a high carrier incidence of pathogenic types prevails at a time when viral infections of the respiratory tract are epidemic, epidemic pneumococcal pneumonia is liable to occur also (Hodges and MacLeod, 1946).

Chance would appear to determine whether the nonimmune individual becomes a carrier of one or more types of pneumococci (Hodges, MacLeod and Bernhard, 1946), although the immune person is less capable of becoming a carrier than the non-immune (MacLeod et al., 1945).

Most of the reported epidemics of pneumococcal pneumonia have occurred in relatively closed communities such as mental hospitals, prisons and military installations. The living conditions in such circumstances appear to favor the dissemination of the more pathogenic types once the latter are introduced into the community. In addition, the incidence of pneumococcal pneumonia is higher in workers in certain occupations such as in steel mills and in coal mines than in the general population (Heffron, 1939).

There is evidence that the normal carrier is of more importance in the dissemination of the infective types than the patient ill with pneumonia (MacLeod et al., 1945).

### THE CONTROL OF PNEUMOCOCCAL PNEUMONIA

From the observations cited in relation to the epidemiology of pneumococcal pneumonia, it seems likely that control can be achieved either by preventing the nonbacterial respiratory infections which predispose or else by specific prophylaxis of pneumococcal infections themselves. A certain amount of success has been achieved through both approaches.

In recent years evidence has been presented that the incidence of influenza can be greatly reduced by immunization with vaccines of influenza virus types A and B, provided the strains of virus present in the vaccine are closely related immunologically to the strains of virus causing epidemic influenza. Improvement in influenza vaccines and their general use may be expected, therefore, to cause a reduction not only in influenza but in pneumococcal pneumonia which occurs with considerable frequency secondary to influenza.

Less success has attended attempts at the control of other nonbacterial respiratory infections grouped together under the general term "common colds," and little progress in this direction can be expected until the agents which cause common colds have been isolated and studied immunologically.

Repeated attempts have been made to immunize against pneumococcal pneumonia during the last 35 years (for a critical review see Heffron, 1939). In the earlier trials, the vaccines consisted of heat-killed pneumococci which were injected subcutaneously. Although proof was lacking, the general opinion of those who employed whole bacterial vaccines was that a beneficial result was obtained. The evidence of Lister and Ordman (1935) in South Africa was especially suggestive of a prophylactic effect. In more recent years, preparations of the capsular polysaccharides have been used following the demonstration by Francis and Tillett in 1930 that the isolated polysaccharides are antigenic for man. Again suggestive evidence was obtained especially through the studies of Felton (Ekwurzel et al., 1938), that immunization of man with the capsular polysaccharides will prevent pneumococcal pneumonia. Most of the studies on antipneumococcal immunization have been deficient in one or more respects, especially because of failure to determine whether the apparent reduction in pneumonia was confined to the types against which immunization was practised, as well

as failure to design the experiment so that adequate controls were included.

Most of the deficiencies inherent in previous attempts at immunization against pneumococcal pneumonia appear to have been avoided in the studies reported by MacLeod, Hodges, Heidelberger and Bernhard (1945), who have presented what may be considered as reasonably conclusive evidence that immunization by subcutaneous injection of purified capsular polysaccharides will prevent pneumococcal pneumonia.

Immunization was carried out in an Army camp where Types I, II, IV, V, VII and XII pneumococcal pneumonia had been epidemic for two years. Accordingly 0.06 mg. of each of the capsular polysaccharides of pneumococcus Types I, II, V and VII was injected subcutaneously into half the population, the remainder serving as controls. In the immunized men, pneumonia caused by Types I, II, V and VII ceased occurring within two weeks after immunization, but continued to occur in the non-immunized controls. The incidence of pneu-

monia caused by pneumococcal types other than those represented in the vaccine was not affected in either group.

Although Types I, II, V and VII pneumococci continued to cause disease in the nonimmune group, the incidence was not so high as was expected on the basis of the previous 2 years' experience, and moreover, the incidence of pneumonia caused by other types was unaffected. It is likely, therefore, that immunization of one-half the population protects not only those who are immunized but also affords a measure of protection to the nonimmune segment. Partial protection of the nonimmune portion of the population may possibly be explained by the observation that the immunized individual is less capable of carrying homologous pneumococci in the pharynx than the nonimmune; and, because of the reduction in carriers, dissemination of pneumococci is greatly reduced.

Large scale immunization of a civilian population has not yet been employed for the prevention of pneumococcal pneumonia.

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# 11

## The Streptococci

### INTRODUCTION

Streptococci belong to the family Lactobacteriaceae; to the Tribe I Streptococceae; to the genus *Streptococcus*. Their common denominator is a Gram-positive micro-organism, spherical or elliptical in shape, and which is not soluble in bile. The units occur in chains, sometimes in pairs, but never in packets. They divide at right angles to the long axis of the chains, which may contain from two to several hundred cocci. Some form capsules, many do not. The discoid colonies on solid media show various contours. Some strains elaborate pigment. The action on blood varies from species to species, and comprises a primary factor which conditions classification. Various carbohydrates are split with the production of acid. Most strains fail to liquefy gelatin. Most are aerobic and facultatively anaerobic, but some are obligate anaerobes.

There are widely distributed in nature many species of streptococci: some are predominantly pathogenic, others are usually saprophytic; but among the latter, several have potential pathogenicity, and a minority none. Some of these play a useful role in industry. Streptococci may also serve as test objects for detecting biologic or chemical reagents.

Aerobic streptococci are separable into four divisions (Sherman, 1937): hemolytic, viridans, enterococci, and milk-souring species (*Str. lactis*). The members of each division have several common physiologic characteristics. Some viridans strains and *Str. lactis* have no known disease-inducing capacities, but are useful in the dairy industry. In general, the hemolytic species are the most pathogenic. Among the serologically identifiable groups some are predominantly disease inducing in

man, others in the lower animals; and the latter only occasionally infect man.

Streptococci cause a wide variety of diseases in both man and animals; but only a few of these infections show specific manifestations because other bacteria often induce similar pathologic pictures. For this reason and also because of the ubiquity of saprophytic streptococci, exact bacteriologic diagnosis is usually advisable.

### HISTORY

Billroth (1874) described globular micro-organisms in chains as complicating factors in wound infections and erysipelas, but argued against their causative role in these diseases. Pasteur (1879) demonstrated similar micro-organisms in puerperal sepsis; and concurrently Ogston (1881) recorded experiments in which pus-containing "micrococci in chains" from acute abscesses led to abscess formation, bacteremia and death when injected into mice and guinea pigs. He furthermore induced the same pathologic phenomena with similar micrococci grown both on artificial media and in eggs.

Robert Koch (1881) described and photographed these microbial forms in all excised erysipelas lesions; and Fehleisen (1882-1883) grew streptococci in pure cultures from such areas, and with pure subcultures induced typical erysipelas in human beings; hence the name *Streptococcus erysipelatis* (Fehleisen, 1882). For a time



they were considered unique specific etiologic agents in this disease. Rosenbach (1884), however, found similar cocci in other types of lesions, particularly in those containing pus, and, therefore, named them *Streptococcus pyogenes*, a nosologic term which has persisted. Early in this century P. H. Hiss (1902) introduced differential tests based upon the ability of different strains to ferment certain carbohydrates; and Andrewes and Horder (1906) set up a system of classification based upon these capacities. This has been variously modified by subsequent investigations, but many of the terms introduced by them have persisted.

The differentiation of streptococci into hemolytic, green or indifferent strains based upon their action on red blood cells in vitro was introduced by Schottmüller (1903); and this differential technic was later elaborated by Brown (1919), who introduced the terms alpha, beta, and gamma to describe slight hemolysis with greening, frank hemolysis, or no effect on erythrocytes respectively. Schottmüller recognized that the hemolytic strains were potentially more virulent than the nonhemolytic varieties. Holman (1916) developed a system of classification combining the action of streptococci on blood and on certain carbohydrates. These systems have mainly historic interest because, in general, there is little parallelism between carbohydrate fermentation and disease-inducing capacities; although, not infrequently, sufficiently close similarity exists among the biochemical properties of certain strains to relate them by these properties.

From the standpoint of disease-inducing capacity with respect to various animal species, the system of classifying streptococci into immunologic groups introduced by Lancefield (1928) has proven most useful. Griffith (1934) simultaneously differentiated strains derived from human beings into types by a slide agglutination method; while Lancefield (1933) developed a pre-

cipitin technic for typing members of group A, based upon their ability to produce type-specific protein substances designated "M." She later (1940) discovered a second protein substance, called "T," which parallels, in certain instances, the type specificity indicated by M-anti-M reactions, although in many others the distribution of T among the serologic types has no relationship to the types established in terms of M substances.

While no system of classification has proven entirely comprehensive, that based upon immunochemical components has proven most useful, particularly in the hemolytic strains pathogenic for man and animals. It does not cover many nonhemolytic strains, nor is it applicable to the strictly anaerobic streptococci first designated by Veillon (1893) as *Micrococcus foetidus*.

## MORPHOLOGY

As indicated by its derivation, the word "streptococci" describes berry-shaped microorganisms growing in chains. The individual cocci may be perfectly spheroid but are frequently ovoid, with their long axes parallel to that of the chain. Each coccus is approximately 1 micron in diameter, with considerable variation arising from nutritional factors; but "minute streptococci" are from one-fourth to one-half of this size. When growing under unfavorable conditions, as on dry or old media, streptococci often assume swollen, bizarre diphtheroid forms. The characteristic shapes appear most readily in young, actively growing cultures in well-buffered serum or blood broth, and assume involution forms in senescence.

The cocci divide in a plane perpendicular to the long axis of the chain, in which several cocci may divide almost simultaneously. Because the individual cocci divide into pairs, a diplococcoid appearance of the members of a chain is often quite striking.

The lengths of the chains vary within wide limits. Enterococci form pairs or

double pairs. Many human hemolytic pathogens grow in chains of 8 to 10 members, others much longer. Viridans streptococci show similar variations, but usually tend to form very long chains. One may obtain macroscopically a rough estimate of the length of the chains from the character of the growth in broth: with short chains, the medium is diffusely turbid; with those persistently longer, conglomerates tend to form and occasionally appear like balls of cotton which microscopically show a skeinlike intertwining of the chains. Near the acid agglutination point and with certain concentrations of salts in fluid media there is a tendency to long-chain formation. The demonstration that length of chains may be conditioned by environmental factors, as well as by a natural tendency in certain strains, has negated the descriptive value of the adjectives *longus* and *brevis* used in early descriptions.

Most streptococci are nonmotile. Kobl-muller (1935) and Pownall (1935) have, however, described motile flagellate forms with morphologic and cultural characteristics somewhat similar to those of enterococci.

Capsulation varies among streptococci both in nature and significance. In species which elaborate polysaccharides as type-specific components, notably group B and viridans, *Str. salivarius* and *Str. MG*, the capsular substance is probably comparable with the soluble specific substance of pneumococci. Among capsulated group A strains and group C animal strains, however, the capsules are composed largely of hyaluronic acid, a complex carbohydrate which is apparently nonantigenic, probably because it is a normal component of the animal body. The significance of this substance with respect to virulence is discussed later in the section "hyaluronic acid," page 252.

Capsule formation should be suspected if the colonies on solid media are mucoid. Microscopically capsules are most easily demonstrated in very young actively grow-

ing cultures; and often when present at that time, none may be demonstrated a few hours later, probably because the hyaluronic acid has passed into the medium. Capsules composed of this substance are most easily demonstrable by the India-ink technic, but they also stain with suitable dyes. These capsules are not formed by strains known to produce hyaluronidase.

The capsules formed by *Str. MG* become obvious when these micro-organisms are suspended in the homologous immune serum. This is an example of the "quellung" phenomenon; and the technic for its demonstration should be applied whenever it is known or suspected that the capsular substance is a type-specific carbohydrate.

## GROWTH REQUIREMENTS

Growth tends to be poor on ordinary solid media unless enriched with blood or serum. Usually, a good nutrient agar containing 5 per cent defibrinated blood furnishes the most satisfactory medium for demonstrating characteristic colony forms. Such blood agar should contain only traces of dextrose, because acids formed from sugar may mask the characteristic action of streptococci on blood, an important factor in their primary classification. As a fluid medium, Todd-Hewitt broth containing the necessary salts, small amounts of dextrose, and enough buffers to neutralize growth-inhibiting acids is satisfactory for most purposes. Streptococci may grow well in a given medium and still form little type-specific M or other immunologically important components; hence amount and rate of growth are not necessarily sufficient criteria of a given medium's efficacy.

The nutritive requirements of streptococci vary widely among the different species. The hemolytic human pathogens are the most exacting, and some of the viridans strains the least. Several media, containing known quantities of amino acids, carbohydrates, inorganic salts and vitamins, have



been tested for their growth-sustaining capacity. Woolley and Hutchings (1940) found that the simplest effective amino acid mixtures for growing groups D and B streptococci contained tryptophane, glutamic acid, isoleucine, lysine, arginine, tyrosine, and cystine, together with glucose, inorganic salts and the vitamins listed in Table 26. Glutamic acid and tryptophane appear absolutely requisite. Other amino acids may replace one another to a certain extent. A reagent having some reducing capacity is necessary; and for group A streptococci purines markedly enhance growth. A readily available source of energy such as sugar is needed; and, other conditions being equal, the amount of growth within certain limits is proportional to the sugar content of the medium. When the acid formed therefrom reaches a given concentration, growth ceases, but if this acid is neutralized, additional growth occurs. An atmosphere containing CO<sub>2</sub> favors the utilization of nutritive materials.

The necessary vitamins are all water soluble, and the different streptococcal species vary in their requirement of these growth-accessory substances (Table 26). A water-soluble substance designated "strepogenin" by Sprince and Woolley (1944) has been found necessary for the growth of group A streptococci. It also enhances markedly the rate of growth of enterococci, and of *Lactobacillus casei*, and has growth-stimulating properties for mice. Woolley has shown that strepogenin is probably a peptide which occurs in highest concentrations in watery liver extracts. It is, however, also present in other tissues.

While investigations of the substances essential for growth of streptococci have provided much valuable information, they are still incomplete in that the emphasis has been largely on growth-sustaining or growth-stimulating factors; and comparatively little work has been done to determine the substances necessary for the anab-

TABLE 26. VITAMIN REQUIREMENTS OF SOME OF THE STREPTOCOCCI \*

SPECIES OR VARIETY OR COMMON GROUP NAME	SERO- LOGIC GROUP	BIOTIN	PANTO- THENIC ACID	NICO- TINIC ACID	THIA- MINE	RIBO- FLAVIN	PYRI- DOXAL	FOLIC ACID	"STREPO- GENIN"
<i>Streptococcus pyogenes</i> . . .	A	+	+	+	+	+	+	±	+
Group F . . . . .	F	+	+	+	+	+	+	+	-
Group G (minute) . . . . .	G	+	+	+	+	+	+	+	-
Group G (large colony) . . .	G	+	+	+	+	+	+	-	-
"Human C" and "Animal C" . . . . .	C	+	+	+	+	+	+	-	-
<i>Streptococcus mastitidis</i> . . .	B	+	+	+	+	- †	+	-	-
<i>Streptococcus fecalis</i> and other "enterococci" . . . . .	D	+	+	+	-	+ †	+	±	-
<i>Streptococcus lactis</i> . . . . .	N	+	+	+	+ †	±	-	-	-
<i>Streptococcus salivarius</i> . . .	-	+	+	+	+	+	-	-	-
<i>Streptococcus equinus</i> . . . . .	-	+	+	+	+	-	-	-	-
<i>Streptococcus bovis</i> . . . . .	-	+	-	-	+	-	-	-	-

\* This table was supplied through personal communication by Dr. J. M. Sherman, who gathered the data both from the literature and investigations in his laboratory.  
† A few strains give the opposite result.  
No streptococcus, the nutritive requirements of which have been determined, has required choline, inositol or para-aminobenzoic acid.

olism of the various immunogenic components of these micro-organisms.

The pathogenic forms grow best at about 37° C. Forty to 42° is the upper limit for most hemolytic pathogens, but enterococci (group D) and many viridans strains grow well at 45° C. Growth of hemolytic and most viridans strains is usually poor below 30° C. and negligible below 20° C., while enterococci and *Str. lactis* grow easily at 10° C.

Most streptococci are facultative anaerobes, but certain strains are obligate anaerobes. The latter occur in surgical infections, sometimes in puerperal sepsis. Anaerobic strains usually have a foul odor, due to the formation of gas containing SO<sub>2</sub>; but there are strains requiring little or no free oxygen in the medium which remain odor free after long incubation.

## COLONY FORMS

On the surface of agar containing blood or serum, streptococci usually grow in disk-like colonies, 1 to 2 millimeters in diameter; but a well-isolated colony may be two or more times larger. Minute streptococci of groups F and G, as well as some members of groups E and M, form tiny colonies. Many different colonial forms have been described: some checkerlike; others spheroid, like sections of a globe; others with craters or central nipples. Many members of group A, when grown on moist blood agar in a well-sealed container, form mucoid colonies which on drying have slightly raised margins and a surface with numerous tiny elevations.

Variants of the same strain may show different colony forms. Among group A strains the differentiation is into matt and glossy colonies. The former, when examined microscopically, have a very finely granular, nonglistening surface, which resembles that of a nonglossy photographic print. The glossy colonies, on the other hand, are small and have a relatively dry glistening surface like a shiny photograph.

Such glossiness is to be clearly distinguished from the dewdrop or mucoid appearance characteristic of the strains which form capsules composed of hyaluronic acid, and also from those viridans strains which polymerize simpler to more complex carbohydrates, for example, sucrose to dextran; the latter strains form extremely mucoid colonies on sucrose agar.

Because considerable confusion exists concerning the significance of the phenomena of mattness and glossiness, this subject requires elucidation. Todd (1927) observed that variants of a strain which were mouse virulent grew in matt colonies, while those growing in glossy forms were avirulent. Because in the case of most other bacteria "roughness" in colony formation indicated avirulence and "smoothness" virulence, the new terms "matt" and "glossy" were introduced. Subsequently Lancefield and Todd (1928) showed that the matt variants of a given strain produced the largest amounts of the type-specific M protein (the letter "M" was adopted from the first letter of "matt"); while the glossy variants produced little or none. Thus, matt colonies, M protein production, and virulence assumed synonymous significance; in contrast, glossy, little M-production and avirulence had similar meanings. It was soon shown, however, that a variant might grow in matt colonies, elaborate fair amounts of M substance and still have low virulence for mice; hence the term "matt avirulent." Thus, virulence, in so far as it concerned mice, was removed from significance with respect to the other two factors, although the possibility remained that it might have such significance with respect to man. Further study of many additional types soon indicated that, within limits, certain virulent strains producing large amounts of M antigen showed colony forms with surfaces closely resembling the glossy avirulent variants of other strains. It thus became obvious that synonymous implications of these three terms had only limited validity. This



led to the unfortunate substitution of the meaning of the word matt to indicate abundant M-antigen production by the strain or variant in question, and glossy little or none, in place of the ordinary meaning of these words. Obviously, to demonstrate this antigenic function, serologic analysis is requisite. Terms ordinarily having visual significance were substituted for those having metabolic or special functional meaning. It would probably have been better to employ the letter M to indicate the elaboration of M antigen by a given strain and G to designate an absence of that function regardless of the visual appearance of the colony; and the author of this chapter suggests this practice in the future, just as the terms R and S are now often used for rough and smooth. Otherwise it seems advisable to qualify matt by the adjective "physiologic" or "metabolic" just as it has been found necessary to append the terms "virulent" and "avirulent" after the word matt.

In spite of the above qualifications concerning the significance of these terms, changes in the appearance of colonies can yield valuable information: decreasing visual mattness and increasing glossiness in the appearance of cultures made week after week following an acute streptococcal sore throat often run parallel with decreasing ability of the strains recovered to produce M antigen together with increasing susceptibility to phagocytosis by normal human leukocytes (Rothbard, 1945). Varying susceptibility to such phagocytosis is probably the best in-vitro technic available for measuring the virulence of streptococci for man. Visual glossiness in strains carried in the nasopharynx of convalescent or recovered persons for long periods after acute streptococcal infections is also suggestive of their relative avirulence; a suggestion that may be tested by the phagocytic technic. Moreover, it should not be forgotten that increasing the virulence of relatively avirulent strains by mouse passage is prac-

tically always accompanied by increasing visual mattness of the passed strains.

Slight alterations in the composition of the culture medium on which streptococci grow may markedly influence their capacity to show visual mattness or glossiness; hence it may be necessary to test the medium with known matt colony variants when in doubt concerning the significance of colony forms.

## ANTIGENIC CONSTITUENTS

Formerly most in vitro studies of streptococci were concerned with their action on more or less clearly defined chemical agents incorporated in culture media. The recent tendency, on the other hand, has been to determine their elaboration of immunochemical components and other substances of possible pathogenic significance. Streptococcal enzymes which may act on bacterial metabolites or attack animal tissues are attracting increasing attention. Following are described the various immunochemical components upon which the serologic classification of streptococci is based. Subsequently certain biochemical and biophysical characteristics are discussed, and correlations among immunologic and physiologic factors are considered.

### C SUBSTANCE AND SEROLOGIC GROUPS

Many streptococci elaborate a carbohydrate designated C. The serologic character of this substance from each group is the factor upon which the division of streptococci into immunologic groups depends.\* Serologic grouping is significant in part

\* At present, immunologic grouping of streptococci is based upon their production of a group-specific carbohydrate C. With many streptococci such production has not been demonstrated; hence they have not been classifiable into immunologic groups. As discussed below, many groups are found divisible into types based upon the production of specific protein or polysaccharide components. When, as among viridans strains, a common substance is demonstrable by serologic technics, the serologic relationships so indicated are probably indicative of type specificity because they parallel agglutination reactions.

because the pathogenic functions of a given strain for a particular animal species are correlated to a considerable degree with the group to which it belongs, although other factors also play a role. The most characteristic sources of the several streptococcal groups so far identified are set out in Table 27. While the different groups are usually encountered under the conditions tabulated, streptococci may find conditions suitable for existence in other environments where they may even be pathogenic. The usual, as well as the occasional, pathogenicity of members of the various serologic

groups for different animal species is shown in Table 30. The assumption of pathogenicity by streptococci in an unusual environment is often favored by prior physical or infectious traumata. Here the streptococci act as secondary invaders.

The ability to classify streptococci serologically has more than academic interest, because, although the members of the various groups are more often found under special conditions, they also occur in other environments. The occurrence of streptococci in milk has various implications: groups E and N are normally present with-

TABLE 27. STREPTOCOCCI WHICH ELABORATE GROUP-SPECIFIC CARBOHYDRATE

GROUP	COMMON NAME	USUAL HABITAT	USUAL PATHOGENICITY	NUMBER OF TYPES	TYPE-SPECIFIC COMPONENT
A	Human <i>Str. pyogenes</i>	Man	Many human diseases	40+	Protein M
B	<i>Str. agalactiae</i> ( <i>Str. mastitidis</i> )	Cattle	Mastitis	6 main 15 + subtypes	Polysaccharide
C	<i>Str. equi</i>	Horse	Strangles	1	Probably protein
	Animal <i>Str. pyogenes</i>	Many animals	Many animal diseases	2+	
	Human C	Man and animals	Respiratory and other infections	8±	
	<i>Str. dysgalactiae</i>	Cattle	Mastitis	?	
E	Group E	Normal milk	None	?	Probably polysaccharide
F	Group F (minute)	Man	Slight; respiratory tract	4	
G	Group G (minute) Group G (large colony)	Man Man Dogs	Upper respiratory tract Many areas Genital and respiratory tracts	1 Several	Probably polysaccharide
H	Group H	Man	Questionable; respiratory tract	?	
K	Group K	Man	Questionable; respiratory tract	?	Probably polysaccharide
L	Group L	Dogs	Genital tract	?	
M	Group M	Dogs	Respiratory tract	?	
D	Enterococci:	Intestinal contents			
	<i>Str. faecalis</i>	Man, many animals and dairy products	Genito-urinary tract, gastro-intestinal tract, abscesses, heart valves, wounds, contaminated food poisoning	Several	
	<i>Str. liquefaciens</i>				
	<i>Str. zymogenes</i>				
	<i>Str. durans</i>				
N	<i>Str. lactis</i> :				
	<i>Str. lactis</i>	Milk	None	?	
	<i>Str. cremoris</i>	Cream	None	?	

Groups A to E were described by Lancefield (1933); groups F and G by Lancefield and Hare (1935); groups H and K by Hare (1935); and groups L and M by Fry (1939); *Streptococcus lactis* (group N) was identified independently by Shattock (1937), Sherman et al. (1940), and Seeleman and Nottbohm (1940). Seeleman and Nottbohm assigned the letter L to this group as they were not acquainted with Fry's appropriation of the letters L and M for the streptococci isolated from dogs. Because of Fry's work, Shattock and Mattick (1943) eventually assigned the letter N to this group; and this designation is now almost universally accepted.



out suggesting any pathogenicity; groups B, "animal" C and *Str. dysaglaetiae*, though present, may have little import—on the other hand, they may have arisen from cows suffering from mastitis which is usually caused by members of these groups; and milk containing them is comparatively innocuous for man. Enterococci and various viridans strains often occur in milk and have usually little pathogenic significance. When, however, milk contains group A streptococci, which may have been derived either from a cow infected with these microorganisms or from a milk handler, its consumption by humans is usually dangerous. Indeed, most milk-borne streptococcal epidemics originate from such sources.

Although, by means of the group-carbohydrate C substances and their corresponding antibodies, it is possible to arrange streptococci in serologic groups, no other biologic phenomena depending upon the existence of the carbohydrate C are known. No group-specific agglutination of streptococci has been demonstrated; nor is there any relationship between the amount of anti-C precipitin in an immune serum and its non-type-specific agglutinating capacity. Furthermore, there is no demonstrable relationship between the amount of C substance various strains produce and their virulence. In fact, in only rare strains have any quantitative differences in the production of C substance been observed within any given group.

The group-carbohydrate C forms a part of streptococcal cells from which it can be separated by some such method as grinding in a ball mill or extracting the bacteria with either strong acids or alkalis. In such solutions it is a nontoxic and nonantigenic haptin. It may, however, be extracted in an antigenic form by a gentle process which leaves it combined with a cellular nucleoprotein. Water-clear extracts form precipitates when mixed with serum containing group-specific precipitins from animals immunized with whole bacteria. When such

mixtures are made in capillary pipettes, only small amounts of the two components are required; hence group testing is practical and relatively inexpensive when suitable sera are available. Indeed, the primary classification of streptococci by this technic is a great saving in time and materials.

Streptococci of each group produce C precipitinogens which are specific for the respective group, and the occurrence of cross-precipitin reactions with sera heterologous to the group under consideration is usually due to other antigenic components common to the groups in question.

#### TYPE SPECIFICITY AMONG VARIOUS GROUPS OF STREPTOCOCCI

Several streptococcal groups are divisible into serologic types. In the case of group A, type specificity depends upon the protection, either active or passive, of an animal with antibodies which are specific with respect to the infecting strains. Therefore, type specificity depends upon an antigenic component common to the members of that type. Such components vary chemically among the different groups: in group A they are proteins and in group C probably proteins; in group B they are polysaccharides, and the same probably holds for groups D, E, F and G. Because most streptococci pathogenic for man fall into group A, the discussion of types and type-specific components is limited mainly to that group.

#### M AND T COMPONENTS OF GROUP A

At least 40 serologic types have been identified among group A streptococci, and there are probably many more. Most group A streptococci elaborate two antigenic components, designated respectively M and T, which have had type-specific implications. This duality has complicated the study of type specificity when agglutination technics were employed; but since these two antigens have been extracted separately from

streptococcal cells, their respective chemical and immunologic properties have been determined (Lancefield and Dole, 1946). They are shown in Table 28.

Glossy colony avirulent variants, on the other hand, produce little or no M substance. They may, however, form mucoid colonies. Animals receiving them paren-

TABLE 28. COMPARISON OF PROPERTIES OF M AND T ANTIGENS OF GROUP A HEMOLYTIC STREPTOCOCCI \*

METHOD OF DIFFERENTIATION	T ANTIGENS	M ANTIGENS
Extraction procedure...	Proteolytic digestion	Heating at pH 2 to 3
Effect of proteolytic enzymes...	Resist digestion	Rapidly digested
Chemical composition.....	Protein, immunologically active substance prepared free of nucleic acid. Not an alcohol soluble protein	Protein (probably independent of nucleic acid): belongs to class of alcohol soluble proteins
Isoelectric point.....	Close to pH 4.50	Not known
Heat	Labile	Stable
Occurrence	Constant component of a given strain, present in both matt and glossy variants	Variable cellular component, constant in certain strains and readily lost in others. Regained on mouse passage of cultures. Present in matt variants only
Specificity	One T antigen may be common to several types; another may be restricted to a single type	Distinct M antigen for each type
Antigenicity and relationship to protection	Highly antigenic both in the intact cell and in solution. Antibodies not protective	Moderately antigenic in the intact cell. Poorly antigenic in solution. Antibodies confer type-specific protection
Relationship to virulence.....	None known. Present in both virulent and nonvirulent forms	One of the essential factors, but additional unknown factors apparently necessary

\* Lancefield, R. C., and Dole, V. P., 1946, The properties of T antigens extracted from group A hemolytic streptococci. *Journal of Experimental Medicine*, 84, 449-471.

The type-specific protein M is closely associated with the virulence of group A streptococci; and the antibodies induced by it in animals are closely related to the type-specific protective action of immune sera. It occurs chiefly in those variants which form mucoid or matt colonies. While strains forming matt colonies may be either virulent or nonvirulent with reference to particular animal species, both matt variants may elaborate approximately equal quantities of M antigen; and animals immunized with either variant have in their sera both type-specific anti-M precipitins and protective antibodies. Such animals are actively immune against infections with many lethal doses of virulent streptococci of the homologous type, but not against strains of heterologous types.

terally produce in their sera neither protective antibodies nor anti-M precipitins; nor do they resist inoculation with virulent variants of the homologous types.

Often after repeated transplantation on artificial media or while living on the mucous membranes or in the tissues of chronic carriers, hemolytic streptococci tend to deteriorate from matt to glossy and simultaneously lose their virulence and their capacity to produce M substance. Such glossy avirulent variants may be made virulent by repeated animal passage, after which they again grow in matt colonies and elaborate much larger amounts of M substance than did the glossy forms (Rothbard and Watson, 1948). Virulent matt variants resist phagocytosis by normal human leukocytes, which easily engulf avirulent streptococci.



Antibodies against the M substance may be determined as follows:

(1) By precipitin tests with suitable streptococcal extracts and precipitating serum from an animal immunized with the specific strain; the serum must be properly absorbed to remove substances which may give confusing non-type-specific precipitates. Such tests can be accurately performed in capillary pipettes (Swift, Wilson and Lancefield, 1943).

(2) By agglutination reactions with suitable diffuse suspensions (Griffith, 1934). Non-type-specific agglutinins directed against other cellular components may give confusing results unless they are properly absorbed from the testing sera. The possibility of cross-agglutinating reactions due to common T antigens and antibodies must also be considered (Lancefield, 1940).

(3) By bacteriostatic tests in which the type-specific antibody renders virulent homologous strains susceptible to phagocytosis by normal human leukocytes (Rothbard, 1945 and Kuttner et al., 1944).

(4) By passive protection tests—the parallelism between anti-M content of a serum and its type-specific protection capacity makes possible the testing for anti-M antibody by means of passive protection of animals with type-specific sera. Sera affording marked passive protection usually contain large amounts of type-specific antibodies which are demonstrable in vitro (Dochez et al., 1919).

Certain chemical characteristics of the M substance make it fairly easily separable from the streptococcal cell and from the T substance. Its relative heat and acid resistance place it in the class of acid soluble proteins.

Crude extracts, made by boiling the streptococcal cells with HCl at a pH of from 2 to 2.5, discarding the cellular residue and most of the non-type-specific proteins precipitated by neutralization, contain both the group-specific C substance, and the type-specific M antigen but not the T substance. Such extracts may be used first in group-specific C precipitin tests. If precipitin reactions occur with group A sera, the same extract may be further tested with the properly absorbed typing sera.

The M protein, separated chemically from streptococcal cells, is a poor antigen; hence is relatively ineffective for actively immunizing animals or for producing anti-M precipitating serum.

A second antigen which is closely connected with agglutination reaction of group

A streptococci and consequently of considerable importance is the T substance. It is a protein having several properties contrasting strongly with those of the M antigen, and which permit a clear differentiation between the two (Table 28). T substance confused the classification in vitro of many group A streptococci until its nature was well defined by Lancefield (1940).

It is more intimately bound to streptococcal cells than is the M substance, but may be released from them by exposure to pepsin or trypsin. Relatively long exposure probably digests most other cellular components, from which the T substance is thereby separated and made soluble. While the T antigen is usually demonstrated in agglutination reactions, extracts of it give precipitin reactions with properly absorbed immune serum. It is destroyed at acid reactions in strong salt concentrations; it resists heating near the neutral point and in physiological solutions, but enzymatic extraction is more effective. The isoelectric point of purified T antigen is about pH 4.5.

The T substance is the most powerful antigen thus far extracted from streptococcal cells. Contrasted with the antigenic properties of the carbohydrate C and the M protein in solution, it induces the formation of antibodies in the serum of rabbits after one or two weeks' immunization; and the same rapidity is observed by immunizing with intact cells. These antibodies have no known protective action; neither has T substance any demonstrable relationship to either pathogenicity or virulence of streptococci. Certain group A streptococcal strains elaborate M antigens as type-specific components, but no T; others both M and T antigens; and still others T antigen alone (Lancefield et al., 1944).

In some types the M and T antigens are both specific for that particular type. The original classification of many streptococci into types was accomplished by Griffith (1934) with the slide agglutination technic without knowledge of the antigen-antibody

systems which were concerned in the agglutination. It developed later that among group A, with a single exception, his type differentiations actually depended on the M-anti-M system. This exception comprised the types designated by Griffith as 10 and 12, which subsequently were shown to contain a common M substance; hence they are now considered as a single type (Watson and Lancefield, 1944). Since knowledge of the T substance has become available, it has been shown that Griffith's differentiation of these two types was based upon the T-anti-T system rather than upon the M-anti-M system which is now accepted as the basis for in vitro type classification of group A streptococci.

Very important with respect to classifying group A streptococci is the fact that some types, which produce their own type-specific M substance, elaborate T antigens which are closely related to those produced by several other types. Two such series of streptococci have been studied in detail: the first comprises types 4, 24, 26, 28, 29 and 46; the second, types 15, 17, 19, 23, 30 and 47 (Stewart et al., 1944). Any member of one of these series will cross agglutinate with serum prepared against any other member of that series; hence, to identify the serologic type of individual members of these two series by the agglutination reaction, all T antibodies must be specifically absorbed from the sera and only M antibodies must remain to agglutinate organisms of the homologous type. The same information with regard to the type of a strain in one of these series may be obtained by applying any of the methods described for identifying its M substance, such as the precipitin reaction, the "bacteriostatic" test or the passive-protection test. The T antigen content of such strains may be determined by agglutination tests with antisera specifically absorbed so that they contain only T antibodies for each series of related types.

#### NUCLEOPROTEINS, THE P SUBSTANCES

A group of substances, extracted with weak alkalies from streptococcal cells and then precipitated with weak acids, are designated nucleoproteins (Lancefield, 1924). They are probably mixtures of nucleoproteins, nuclein, mucin and other proteins such as globulin. In group A such extracts generally do not contain appreciable amounts of type-specific antigens. Polysaccharide antigens present in the initial extract are separated when the nucleoproteins are precipitated with acid. Antibodies in the sera of animals immunized with whole streptococci or with P extracts cross with other members of the same general class of bacteria: for example, nucleoproteins from hemolytic streptococci react with antisera from animals immunized with nucleoproteins from both hemolytic and nonhemolytic streptococci, or with similar preparations derived from pneumococci, and even to a lesser degree with antisera prepared by immunizing rabbits with staphylococcus nucleoproteins. Precipitin tests with sera from patients who have had such diverse diseases as hemolytic streptococcal infections, endocarditis caused by viridans streptococci, and pneumococcal pneumonia all give positive-complement-fixation reactions with nucleoprotein extracts prepared from streptococci. Furthermore, in testing the skin reactivity of patients who have undergone hemolytic streptococcal infections, positive reactions are induced with extracts from all three general categories of streptococci, namely, hemolytic, green and indifferent; although the most intense reaction is usually with extracts of bacteria corresponding to the general category of streptococci which have infected the patient.

Probably the P substances make up much of the streptococcal cells. Their tendency to cross serologically, together with the difficulty in removing them completely from extracts designed to contain a preponderance of other specific streptococcal compo-



nents, makes it difficult to demonstrate absolute serologic specificity with such extracts. What the pathogenic role of nucleoproteins may be is unknown, because it is difficult to prepare any one of them in a pure enough state to test their possible unique tissue-injuring capacity.

#### STREPTOKINASE (FIBRINOLYSIN)

The phenomenon of lysis of a fibrin clot by streptococcal filtrates and the neutralization of this fibrinolysin by immune serum, which was discovered by Tillett and Garner (1933), has been shown by Christensen (1945) to depend upon two components: (1) a proenzyme designated plasminogen, which is present as an inactive proteolytic factor in all human sera; and (2) an activator, designated streptokinase, which is elaborated by many strains of streptococci. The streptokinase acts catalytically with the plasminogen to form an active proteolytic enzyme called plasmin, which digests fibrin and other proteins. One of the following factors may inhibit this digestion: (1) the presence in the serum of antistreptokinase, a true antibody induced by infection with hemolytic streptococci containing sufficient streptokinase; (2) the occurrence in blood of an antiprotease not related to a specific antibody; (3) a deficiency in the blood of the proenzyme, plasminogen.

Kaplan (1946) has devised a quantitative test for determining the amount of antistreptokinase formed in the sera of patients infected with hemolytic streptococci. Standard fibrinogen solution, clots and purified streptokinase are prepared together with plasminogen of known strength. Antistreptokinase combines with streptokinase in multiple proportions; hence by testing progressive dilutions of the serum under consideration against a standard streptokinase, the amount of antibody may be determined. With a logarithmic scale based on successive fivefold dilutions of serum, a two-tube difference in neutralization indicates a significant difference in the measurable amounts of antistreptokinase.

Complete inhibition of clot solution demonstrated by Tillett and Garner's technic and a level higher than 150 units of antistreptokinase per cubic centimeter of patient's serum by Kaplan's method indicate the existence of a fairly recent hemolytic streptococcal infection. Those streptococcal strains which produce much streptokinase induce the formation of more antistreptokinase in patients than do strains producing little. Absence of this antibody is not, however, valid evidence of absence of a prior hemolytic streptococcal infection because some strains are poor streptokinase producers, and patients probably vary in their antibody responses. In fact, more streptococcal-infected patients develop significant increases of antistreptolysin O in their sera than they do of streptokinase.

Streptokinase is produced by strains of the following groups: A, "human" C and large colony G; very rarely by those of groups B and F (Commission on Acute Respiratory Diseases, 1947). The presence of the proteolytic enzyme plasmin in a patient's blood might conceivably induce demonstrable lesions in his tissues; but no known pathogenic function has been definitely associated with streptokinase. This subject has, however, been little explored; and with the possibility of studying quantitatively this kinase and plasmin and associated inhibitor systems, this topic warrants further investigation.

#### HEMOLYSINS

Streptococcal hemolysins are the substances elaborated by these bacteria which dissolve red blood cells and release hemoglobin into the surrounding medium. They are often called streptolysins, and antibodies against them antistreptolysins. Studies of the nature of the several streptococcal hemolysins were quite confusing until some order was established by Todd (1938, 1939) and his collaborators.

Group A streptococci elaborate two dif-

ferent hemolysins: streptolysins O and S. O indicates oxygen lability, and S indicates serum extractability. Streptolysin O is elaborated by most members of group A streptococci, by the "human" group C strains and by the large colony forms of group G. Streptolysin S, on the other hand, is formed only by members of group A. Hemolytic strains of the other serologic groups produce hemolysins unrelated antigenically to either streptolysin O or S.

Streptolysin O, found in serum-free broth-culture filtrates, is readily oxidized into an inactive form. It has a protein containing —S—S linkages, which, when changed to —SH groups, cause it to become hemolytically active (Herbert and Todd, 1941). Either in the inactive, oxidized form or in the hemolytically active reduced state it combines quantitatively with antistreptolysin O, an antibody found in the sera of man or animals following infection with streptolysin-O-producing strains of streptococci. Animals long immunized with broth filtrates of such streptococcal cultures also produce these antibodies. When neutralized with immune sera, streptolysin O is no longer hemolytic; and this phenomenon forms the basis of a quantitative test for antistreptolysin O. Large amounts in a patient's serum, or progressively increasing titers of this antibody within two to three weeks indicate a recent hemolytic streptococcal infection. About 85 or 90 per cent of the patients so infected develop demonstrable amounts of antistreptolysin O which may persist for months and possibly years. Relatively little is known about the tissue-injuring capacity of streptolysin O because it is lethal for mice or guinea pigs only in large doses; and in such high concentrations it kills the animals quickly with a shock type of death.

Streptolysin S, intimately connected with group A streptococcal cells, is a lipoprotein or closely associated with lipoproteins (Herbert and Todd, 1944). It is easily demonstrable when the streptococci are grown in

broth containing serum and is extractable from the streptococcal cells by means of serum (Weld, 1934). It is also dissolved from streptococcal cells by crude lecithovitelin prepared from egg yolk. It is very unstable and can be kept active only when frozen with solid CO<sub>2</sub> or in lyophilized dialyzed solutions. Antibodies against streptolysin S can be induced in animals only by injection of intact streptococcal cells; therefore, streptolysin S in serum extracts probably exists as a haptin. This substance is very pathogenic for laboratory animals. Those succumbing within 24 or 48 hours after injection show degeneration of the parenchymatous organs and laking of the blood throughout the body (Barnard and Todd, 1940). Okamoto (1940) reported that the production of hemolysin (similar to streptolysin S) is markedly enhanced by adding from 0.5 to 2.0 per cent of nucleic acid to the medium.

Most group A streptococci elaborate both streptolysins O and S. Some, however, produce only streptolysin O, others only streptolysin S.

By comparing the action of these last two varieties, it has been shown that the hemolysis about colonies on the surface of blood agar is due entirely to streptolysin S. Strains producing only streptolysin O may, therefore, seem nonhemolytic, especially in markedly aerobic environments; and special conditions are required for the demonstration of their hemolytic capacity when it depends upon streptolysin O. Patients infected with strains of streptococci which produce no streptolysin O may develop no antistreptolysin O in their sera. While concentrations of antistreptolysin O in the sera of man or animals may be several hundred and occasionally several thousand units per cc., that of antistreptolysin S reaches from only 30 to 50 units. Because the blood of certain animals, particularly that of horses, may naturally contain large amounts of antistreptolysin O, its incorporation into blood agar plates may prevent hemolysis about deep colonies if the streptococci under consideration elaborate only streptolysin O. As most group A hemolytic streptococci also produce streptolysin S, the majority hemolyze



erythrocytes from most species of animals. Because normal rabbit's blood rarely, if ever, contains any antistreptolysin O, it is preferable for preparing blood agar plates to study the hemolytic capacity of streptococci.

The nature of the substance producing so-called "alpha" hemolysis has not been much investigated. In any event, the green or brown pigment formed by viridans strains about colonies or at the bottom of serum blood broth cultures is a more striking phenomenon than is "alpha" hemolysis.

#### ERYTHROGENIC TOXIN

Many streptococci, while growing in broth and also in human tissues, produce a soluble erythrogenic toxin which passes readily through bacteria-retaining filters. In contrast with diphtheria toxin, it resists several hours heating at 60° C., but is destroyed by boiling for one hour. This toxin, when employed in the Dick test (1924), must be diluted at least 100-fold with normal salt solution to eliminate possible skin irritating substances present in the broth used for growing the streptococci; and uninoculated similarly diluted broth controls must be employed. To test the amount of erythrogenic toxin produced by a given strain, increasing dilutions of filtered broth culture, in 0.1 cc. doses, are injected intracutaneously either into susceptible humans, or into young white goats or rabbits of known susceptibility. A positive Dick test consists of an erythematous, often edematous, area more than 10 mm. in diameter, which appears within 6 to 24 hours. In quite sensitive subjects, or with very potent toxins, the erythema may be from 20 to 30 mm. across.

Infants below six months of age usually give negative Dick tests; but thereafter, children show increasing susceptibility until about the fourth year, when it is the highest. Subsequently there is statistically a gradually decreasing susceptibility, and about 80 per cent of adults react negatively. The

observation that infants give positive Dick reactions only when older than 6 months has led to the theory that their susceptibility is a phenomenon of hypersensitivity. Another explanation is that the fetus has acquired *in utero* a certain amount of antitoxic immunity which gradually disappears in the few months succeeding birth, but Cooke (1927, 1928) has shown that the insusceptibility of young infants is unrelated to insusceptibility of the mother to the toxin.

During the first five or six days of scarlet fever practically all patients give positive Dick reactions; but with recovery, the relative number of positive reactions diminishes until practically all tests are negative by the fourth or fifth week after infection.

The erythrogenic toxin produced by different strains of streptococci varies qualitatively. For example, Coffey (1938) found that about 80 per cent of several hundred strains produced a toxin which was neutralized by a single antitoxic serum from animals immunized with strain N.Y.5. The toxin elaborated by most of the remaining 20 per cent were neutralized by sera with one or the other of two additional strains; and the toxins from a small residual minority of strains was neutralizable by a combination of these three sera; hence, there are at least five different erythrogenic toxins. This multiplicity of toxins explains how some patients have repeated attacks of scarlet fever, for the symptoms and signs of this disease derive to a considerable degree from the action of these toxins; and recovery is paralleled by the development of antibodies which neutralize the respective erythrogenic toxin. When infected with a strain producing an erythrogenic toxin not neutralizable by his serum, a patient will probably develop a rash even though he has suffered previously from scarlet fever.

Antitoxic sera are produced by immunizing animals with broth culture filtrates of streptococci, but better by combinations of

toxins and living bacteria. Such sera are useful therapeutically, but for widest application they should be produced with at least five immunologically distinct toxins. The official United States standard for antitoxic sera specifies that 1 cc. must neutralize at least 1,000 skin test doses of toxin.

Persons whose susceptibility to scarlet fever is demonstrated by positive Dick reactions show a reversal of these phenomena after immunization with this toxin. They may, however, be subsequently infected with group A streptococci; hence this artificially induced immunity is antitoxic and not antibacterial in nature. Potent antitoxic sera possess the ability to blanch a scarlatinal rash. This Shultz-Charlton phenomenon (1918), demonstrable only in the first few days of scarlet fever, is due to neutralization of the toxin in the skin. It is occasionally useful in differentiating scarlatinal from other cutaneous erythemas.

Different hemolytic streptococcal strains vary considerably in the amounts of erythrogenic toxin produced. Those cultured from practically all patients with scarlet fever possess this capacity, as do many strains derived from other sources. Most erythrogenic toxin-producing streptococci belong to group A, some to "human" group C, and an occasional one to group G. Other groups are apparently negative in this respect. Some observers have reported that certain types among group A contain more Dick toxin-producing strains than do other types; but such data are usually weighted. The observation that in epidemics of streptococcal infections with strains all belonging to a given type there is a wide variation in the incidence of scarlet fever is explainable on two factors: (1) quantitative differences in the ability of these strains to produce erythrogenic toxin; (2) varying susceptibility to the toxin among different members of the population. Such varying susceptibility is due not only to the presence of some previous sufferers from scarlet fever but also to an antitoxic immunity induced in many

people by repeated infections with strains elaborating minimal amounts of erythrogenic toxin, amounts too small to induce a rash.

## OTHER STREPTOCOCCAL COMPONENTS

### STREPTOCOCCAL PROTEINASE

Under suitable conditions many strains of group A streptococci elaborate into broth cultures a proteolytic enzyme designated streptococcal proteinase (Elliott, 1945).

It is formed as a zymogen or proenzyme which becomes active under favorable conditions. When active, it can digest casein, milk, gelatin, benzoyl-L-arginineamide, human and rabbit fibrin, streptokinase, and the M antigen of group A streptococci. The proenzyme is activated by low concentrations of trypsin, by small amounts of active proteinase, and also autocatalytically under suitable reducing conditions. It remains inactive in broth cultures grown at 22° C. or under markedly aerobic conditions, but becomes active when the temperature is raised to 37° C. The formation of active enzyme is suppressed in broth made with neopeptone, but is favored by most other peptones. This proteinase is probably the same as the histase of Frobisher (1926) which digests meat in boiled meat broth cultures. It is antigenic (Todd, 1947); and when injected for long periods in large amounts into animals, induces the formation of antibodies which inactivate it in vitro. Patients infected with hemolytic streptococci, however, produce very little antiproteinase.

The presence of this proteinase may seriously interfere with the anti-M typing technic. Many avirulent, glossy strains, which apparently produce little M antigen, elaborate large amounts of the proteinase. Virulent, M-producing strains, on the other hand, produce little. Apparently production of proteinase and M are mutually exclusive phenomena, but M antigen may be produced by streptococci and rapidly destroyed by proteinase; therefore, to favor the protection of any M antigen formed it is advisable to employ broth-containing neopeptone, in which medium only the proenzyme is found. Strains apparently producing no M when grown at 37° C. should be retested after being incu-



bated for 24 to 48 hours in broth cultures at 22° C.; under these conditions, even though proenzyme exists, it usually is inactive. The elaboration of proteinase, moreover, by a given strain prevents that strain from producing streptokinase or destroys streptokinase which might be elaborated in cultures of that streptococcus (Rothbard and Todd, 1948).

Streptococcal proteinase has not been shown to have pathogenic capacities, although this subject has been little explored. Nevertheless, conditions *in vivo* may exist where the zymogen in virulent strains may become activated and injurious to tissues.

#### HYALURONIC ACID

Hyaluronic acid is the main component of the capsules formed by many strains of group A and by most animal strains of group C streptococci (Kendall et al., 1937). It also makes up much of the vitreous humor and of Wharton's jelly of the umbilical cord, the interfibrillar material in connective tissues, and the intercellular ground substance of cartilage; and synovial fluid contains large amounts of this complex carbohydrate.

When a fine loop is touched to mucoid colonies of hemolytic streptococci and slowly withdrawn, the mucoid substance follows as a fine thread. In young broth cultures the hyaluronic acid is closely associated with the streptococcal cells, about which it is demonstrable as capsules by the India-ink technic or by special stains. In older cultures it passes into the broth from which it may be recovered by chemical methods. When group C streptococci are treated with hyaluronidase, they become susceptible to phagocytosis; but, because subcultures form capsules, they obviously have not lost their hyaluronic acid-producing capacity.

In group C streptococcal infections of animals a close relationship between hyaluronic-acid production and the virulence of these bacteria seems definite: virulent strains are heavily encapsulated; avirulent ones are usually nonencapsulated. Animals infected with the former recover when treated with testicular extracts or leech head extracts containing large amounts of hyaluronidase (Hirst, 1941).

In the case of group A streptococci, however, even though virulent strains are often heavily encapsulated, a comparable relationship between hyaluronic acid encapsulation and virulence has not been definitely established. In fact neither Hirst nor McLean (1941) could demonstrate any protective action from hyaluronidase treatment of group A infected animals, although Seastone (1939) and Kass and Seastone (1944) have reported positive results with crude testicular extract containing hyaluronidase. Rothbard (1948) has resolved the question by demonstrating that hyaluronidase capsules interfere somewhat with phagocytosis of capsulated group A streptococci; hence to a certain degree these capsules apparently contribute to the virulence of these streptococci. The relationship between M antigen formation and virulence, on the other hand, is quite firmly established and plays a major role in virulence.

Hyaluronic acid is not antigenic, at least no antibodies against it have been demonstrated, even though the animals have received intensive treatment with this substance. This lack of antigenicity is not surprising in view of the normally large amount of this substance in the animal body. The fact that it forms such an important component of connective tissues has led to a hypothesis that its alteration *in vivo* might be one of the injurious manifestations of streptococcal infections, but this hypothesis has received no experimental support.

#### HYALURONIDASE

This specific streptococcal enzyme elaborated by only certain strains breaks hyaluronic acid into simpler components having a nonmucoid character. The capsules on streptococci are quickly removed by treatment with extracts from various sources containing hyaluronidase.

Hyaluronidase is probably an important constituent of the so-called "spreading factor," a substance which, when introduced into the tissues, breaks down their capacity to localize foreign substances to a small area (Duran-Reynals, 1933, 1942). The







mechanism of this breakdown supposedly rests upon the ability of hyaluronidase to attack the hyaluronic acid in the tissues. Because of this action, hyaluronidase is considered one factor which hypothetically enhances bacterial virulence. Among group A streptococci, no parallelism between hyaluronidase production and virulence has been demonstrated. Indeed, the formation of hyaluronidase and hyaluronic acid by a given strain of streptococci are mutually exclusive phenomena. Virulent group A strains do not produce this antigen and are heavily encapsulated. Among group C streptococci, moreover, those strains forming the largest amount of hyaluronic acid are the most virulent. Crowley (1944), in examining 308 group A strains, found hyaluronidase activity only among members of types 4 and 22; while among 68 strains belonging to groups C and G, 48 formed considerable amounts of this substance. Thus, most experimental data apparently exclude any direct relationship between streptococcal virulence and hyaluronidase production. It is, however, hypothetically conceivable that under as yet unknown circumstances this enzyme might attack the somatic hyaluronic acid focally and thus induce lesions. More data are required to settle this question (Thomson, 1944).

## PHYSIOLOGIC CHARACTERISTICS OF STREPTOCOCCI

### BIOCHEMICAL CLASSIFICATION

Long before serologic methods for classifying streptococci were introduced, the physiologic activities of these bacteria had been extensively studied. Many correlated traits were disclosed and numerous descriptive adjectives introduced. In Bergey's Manual (1948) are listed 16 recognized and 6 provisional species of aerobic streptococci; also 8 anaerobic species, all based on physiologic differentiation. There are also appended names of over 100 other varieties.

The purpose of classifying streptococci is to determine: (1) their natural habitat; (2) their disease-inducing propensities; (3) their possible deleterious action on foods and other substances; and (4) their useful capacities in commerce. A study of classification is obviously justified from the viewpoint of pure science.

Following the development of serologic methods for classifying streptococci, several investigators have correlated their results with data furnished by physiologic procedures. Some of this information has been summarized in Table 29, which follows many suggestions made by Sherman. No attempt is made in this table to show all of the recorded biochemical reactions of the streptococci listed. In originally preparing this table most of the recorded biochemical reactions were listed, then those chiefly having differential significance were retained. The physiologic and biochemical traits shown in that table as well as others of a similar nature are not to be considered as absolute in their significance. To determine them, great technical exactness is requisite: the degree of purity of the critical substances added to media must be known; media held long in storage often give inaccurate results; differing times of incubation may yield variable end reactions. Variants which have lost one or more enzymes may appear in cultures. In spite of these objections, the characteristics presented have validity in the majority of instances, and it is usually a combination of traits, rather than one, that is diagnostically significant.

By employing a combination of functions possessed in common by members of each of 4 divisions, it appears logical to divide streptococci into such divisions (Sherman). While hemolysis is useful as a primary classifying technic, there occur nonhemolytic varieties in many of the serologically differentiated groups which were originally restricted to hemolytic strains. In group B, nearly half the strains are nonhemolytic; there are nonhemolytic subdivisions



of group C, *Str. agalactiae*, and strains causing joint ill of lambs; and in group D, which for several reasons is set off by itself, there are two hemolytic varieties. Thus, hemolysis, like any other bacterial function, has only relative value in classification. It is noteworthy that the ability to elaborate a group-specific carbohydrate does not necessarily correlate closely with some other functions. Indeed, other characteristics seem more cogent in bringing certain divisions together. For example, their inability to grow in milk containing 0.1 per cent methylene blue differentiates the two divisions, hemolytic and viridans, from the enterococci and milk-souring streptococci, and this separation is confirmed by their behavior at certain critical temperatures. There are, in fact, several valid reasons for recognizing these four divisions. While the ability to elaborate a group-specific carbohydrate C might seem to bring groups D and N close to groups A to L (omitting D), other characteristics set them off from those groups.

#### HEMOLYTIC STREPTOCOCCI (DIVISION 1)

With previously noted qualifications, the first division comprises a category in which the members are mainly hemolytic on blood agar and most of which produce soluble hemolysins in serum broth; they also elaborate group-specific C antigens. These groups by and large include most of the pathogenic streptococci. In certain respects they are closely related to division 2, in others quite distinct.

#### VIRIDANS STREPTOCOCCI (DIVISION 2)

These species do not cause beta hemolysis of red blood cells, but some may induce so-called alpha hemolysis; some turn hemoglobin brown or green, while others have no effect on blood. The latter are designated indifferent or gamma streptococci. There are wide variations with respect to greenish

alteration of hemoglobin, in part dependent upon the blood employed and in part upon inherent capacities of the species in question; nevertheless, some species usually produce much green pigment and others little or none.

Viridans streptococci, in contrast to members of commonly recognized serologic groups, have not been found to produce a somatic carbohydrate C by which they may be divided into groups. Solowey (1942) identified several "groups" by precipitin reactions; but subsequent work indicates that the responsible antigenic components more closely resemble the soluble-specific substances of pneumococci than the carbohydrate C of the streptococci. On the basis of these substances, some viridans varieties have been divided into serologic types. One cannot state categorically, however, that viridans strains do not elaborate group-specific antigens, but rather that none has been recognized. In many respects, viridans streptococci resemble pneumococci, from which at times they can be separated only on the basis of bile insolubility. While their action on blood is like that of some members of groups D and N, they appear more closely related to hemolytic streptococci in their reaction to methylene blue as well as in some other physiologic characteristics.

In Table 29, nine different viridans varieties are listed, some apparently comprise distinct species, others are not so clearly defined. Many others have been described, but those listed are the most frequently encountered. *Str. salivarius* can be recovered from many lesions in the mouth or nose and occasionally in the lungs, as well as in apparently normal human mouths and intestines. It frequently causes subacute bacterial endocarditis. It elaborates polysaccharides which help in separating it into a distinct species (Niven et al., 1941). Closely related *Str. mitis*, of similar distribution, comprises a less clearly defined category (Sherman et al., 1943). *Str. SBE* (Washburn et al., 1946), synonym *Str. sanguis*, has been recovered chiefly from the blood of patients with subacute bacterial endocarditis and has not been found in normal human

mouths. There are two distinct serologic types, designated 1 and 2, and a third variety which elaborates both type antigens. This species of streptococci specially resists the bacteriostatic action of penicillin (Loewe et al., 1946). *Str. MG*, while found in normal human mouths, appears to have some symbiotic relationship in many cases of atypical pneumonia. In addition to certain physiologic peculiarities, most characteristic of which are fluorescence of its colonies in ultraviolet light and production of ammonia from peptone, it elaborates a type-specific polysaccharide common only among its members, and another which is closely related to a type-specific polysaccharide produced by *Str. salivarius* (Mirick et al., 1944). *Str. bovis*, a normal inhabitant of bovine intestines, is frequently encountered in the human intestinal tract. Barger has ascribed to it pathogenic capacities in chronic enteritis, hence the name Barger's streptococcus. It occasionally causes subacute bacterial endocarditis. *Str. equinus*, the most common streptococcus in the feces of horses, is also not rare in human intestinal tracts. It has been recovered from inflammatory exudates in the gastrointestinal and genito-urinary tracts of man, but whether as a secondary invader or as a primary pathogen is unknown. *Str. thermophilus* and *Str. acidominimus* have no demonstrable pathogenic capacities, but are important in dairy bacteriology because they resist the degree of heat used for pasteurizing milk; hence, their presence in dairy products may be confusing unless they are identified. The first is used commercially as a starter in making Swiss cheese. The second is characterized by its low acid production and its inability to clot litmus milk. *Str. uberis* (Minett et al., 1929) is placed last in this division because there are differing opinions concerning its position among the streptococci. It occurs in apparently normal milk, and it also induces a mild form of bovine mastitis. It resembles viridans streptococci in the following features: action on blood; failure to grow in milk containing 0.1 per cent methylene blue; in 6.5 per cent NaCl; or at an alkaline reaction of pH 9.6. In several physiologic functions it resembles *Str. agalactiae*, and in some the enterococci and *Str. lactis*. Inability, so far, to demonstrate a definite group-specific carbohydrate among its members, or one related to any of the well-established groups, in the author's opinion, leaves, for the moment, only the viridans division of streptococci in which it can be tentatively placed. Variations among its

members in their action on some carbohydrates, and the demonstration of from 10 to 15 serologic "types" by agglutination technics (Plastring and Williams, 1939) suggest that these streptococci comprise a somewhat heterogeneous category.

While viridans streptococci are, at times, difficult to identify one from the other, certain physiologic traits facilitate the classification of certain species. Reactions on 5 per cent sucrose agar and in 5 per cent sucrose broth reveal the following peculiarities: by synthesizing dextran or levan, salivarius strains produce large mucoid colonies on sucrose agar but have little effect in sucrose broth; *Str. SBE* does not synthesize gummy polysaccharides on aerobic sucrose agar media but does so in sucrose broth as indicated by thickening or jelling of the medium; *Str. MG* forms on sucrose agar small fluorescent colonies and does not thicken sucrose broth. Immune sera, made by immunizing rabbits with these viridans species respectively, facilitates their identification by precipitin reactions provided the sera are properly absorbed.

In their ability to synthesize gummy substances from sucrose, some of the viridans streptococci resemble the slime-producing betacocci (Orla-Jensen), synonym leuconostoc, listed in Bergey (1948). These latter, as emphasized by Orla-Jensen, have their normal habitat on plants and grow best below 30° C.; they always form levulactic acid, and sometimes, in addition, dextrolactic acid. This is in contrast to the streptococci derived from animals or animal products, such as those tabulated, which produce dextrolactic acid. Morphologically the betacocci or leuconostoc resemble viridans streptococci or enterococci, from which it may be necessary to differentiate them.

#### ENTEROCOCCI (DIVISION 3)

Although enterococci are widely distributed in nature and usually occur under circumstances not related to disease, their pathogenic capacities when they act, figuratively speaking, "out of bounds" make them worthy of serious consideration. Their combination of abilities to grow in milk containing 0.1 per cent methylene blue and on 40 per cent bile agar and at temperatures and salt concentrations lethal for most other



streptococci, together with the elaboration of the group D specific carbohydrate, distinguishes them as a definite species in which the four varieties listed in Table 29 may be differentiated (Sherman, 1937).

The hemolytic capacities of *Str. zymogenes* and *Str. durans* might cause confusion with other hemolytic streptococci; but this confusion is immediately eliminated by determining the group characteristics listed above. These two varieties differ from one another in their action on several carbohydrates. *Str. fecalis* and *Str. liquefaciens*, nonhemolytic enterococci, are separable because the latter liquefies gelatin, a very rare occurrence among streptococci. Orla-Jensen (1943) has recorded gelatin-liquefying capacity among many members of group A streptococci under very peculiar circumstances: after 14 to 16 days incubation of gelatin stab cultures, and following the death and autolysis of these micro-organisms. This gelatin-liquefying enzyme is intracellular in origin, in contrast to the one elaborated into the medium by *Str. liquefaciens*.

Enterococci are frequently the causative agents in genito-urinary tract infections, occasionally in diseases of the respiratory tract and accessory organs, sometimes in areas contiguous to or draining the intestines, and occasionally they induce subacute endocarditis. Their relative resistance to sulfonamides and penicillin makes their identification important as a guide to therapy. Because they resist pasteurization and also because some strains produce toxic substances in cheese or custards, they may be potential sources of food poisoning, although they occur as harmless inhabitants in many dairy products. It sometimes becomes necessary to differentiate them from commercially useful *Str. lactis*.

#### STREPTOCOCCUS LACTIS (DIVISION 4)

These streptococci, which have the property of elaborating the group N specific carbohydrate, have no known pathogenic propensities; but their frequent presence in milk, which they readily coagulate, make them important in dairy bacteriology. They

are easily differentiated from enterococci by both physiologic and serologic tests. The two recognized varieties are identified by the few physiologic peculiarities listed in Table 29.

#### IDENTIFICATION OF VARIETIES

The physiologic characteristics listed in Table 29, in addition to making plausible the separation of streptococci into four main divisions, at times also makes it possible to identify a strain as a member of a particular group or species. These peculiarities are emphasized by heavy type, which indicate their value within a given division, but not among strains belonging to different divisions. For example, group B (*Str. agalactiae*), while often nonhemolytic, has traits which render it easily identifiable: many strains produce yellowish or orange-colored pigment specially visible in starch broth or in the sediment of broth cultures; and their resistance to bile, their production of acid having a final pH of 4.2 to 4.8 in 1.0 per cent glucose broth, and their ability to split sodium hippurate comprise a unique combination among the hemolytic streptococci. This group has been divided serologically into six types and over fifteen subtypes, the latter by agglutination technics (Stableforth, 1946). There are different opinions whether some group B strains are exclusively of bovine pathogenicity (*Str. mastitidis*) and others of human origin (human group B). Both varieties have been recovered from both man and cattle; but whether these two mammalian species often cross infect one another with group B streptococci is still undecided. Probably most bovine infections arise from bovine sources. Confusion with group E streptococci might occur; but the small group E colonies with wide zones of hemolysis, together with a failure to produce pigment or to grow on bile agar and the ability of group E streptococci to attack both trehalose and sorbitol, distinguish them quickly.

*Enterococci* and *Str. lactis*, often formerly confused one with the other, are readily identified from the physiologic characteristics shown in Table 29. Their respective identities are confirmed by serologic grouping procedures which eliminate other time-consuming maneuvers. Identification of varieties within these two divisions, however, must be effected by physiologic tests listed in Table 29.

A possible phylogenic relationship among the various groups of streptococci, based upon their physiologic functions, has been noted by Orla-Jensen (1943). They proceed in the following order: group D; group B; group C, "animal," equi, and "human"; finally group A.

The reactions listed in the lower two-thirds of the table are more valuable in identifying members within a particular division than in separating one division from another. Here, as already noted, the reactions set in heavy type emphasize the tests specially useful for identifying the particular streptococcus within its division.

Group C illustrates clearly the relative values of the serologic grouping technic compared with physiologic tests. There are both hemolytic and nonhemolytic subdivisions; and among the former, three distinct varieties are identifiable from their action on trehalose, sorbitol and glycerol. *Str. equi* and *Str. "animal \* pyogenes"* are almost unique among hemolytic streptococci in failing to ferment trehalose and glycerol, and are separable from one another because of the inability of *Str. equi* to ferment sorbitol. While the so-called "human" group C strains are separable from *Str. equi* and the "animal *pyogenes*" strains on the basis of their action on these three carbohydrates, they are easily confused with group A and large colony group G strains, and their definite identification rests upon serologic demonstration of the group C precipitinogen combined with their action on the three carbohydrates above mentioned. The nonhemolytic group C varieties *Str. dysgalactiae* (Minett et al., 1929) would be placed among the viridans streptococci unless their ability to elaborate group C precipitinogen were demonstrated. With serologic technics, several types among group C

\* The designations "animal" group C (synonyms "animal" *Str. pyogenes*, *Str. pyogenes animalis* and *Str. zooepidemicus*) and "human" group C (synonyms *Str. equisimilis* and large colony group C) have crept into the bacteriologic literature but require qualification. Although the 2 varieties are readily differentiated by physiologic tests, their pathogenic capacities with respect to animals and man must be understood. While the "animal" group C strains comprise the streptococci most widely pathogenic for animals and rarely pathogenic for man, the "human" group C strains are also pathogenic for animals as well as for man. Possibly the somewhat clumsy term "large colony group C streptococci" would more exactly describe them, because their colonies are to be contrasted with the small ones of *Str. equi* and the mucoid colonies of most "animal" group C strains. The latter would be accurately described by the term *Str. pyogenes epizooticus*.

strains have been identified (Bazeley and Battle, 1940; Simmons and Keogh, 1940).

In general, those investigators who have used both serologic and physiologic methods for classifying streptococci agree that neither absolutely replaces the other, but that the most satisfactory identification is attained by employing those parts of each which are the most applicable in any given situation.

## DISTRIBUTION AND RANGE OF PATHOGENICITY

Streptococci are widely distributed in nature. Wherever man or animals reside, these micro-organisms may occur either as pathogens, commensals or free-living bacteria; in the latter state they do not derive their sustenance directly from an animal host. Indeed, some species, for example *Str. lactis*, lead their entire existence apart from animal tissues, it is uncertain where. Sherman (1937) suggests it may be on plants. Their ubiquity is shown by the speed with which they contaminate and sour milk exposed to air. The enterococci are also widely distributed, as might be expected from their normal habitat in the intestines of man and the many animals whence they are constantly excreted in the feces. The worldwide use of manure as a fertilizer insures repeated introduction into the soil of enterococci and many other streptococci which may occur in the intestinal tracts of man, beasts and birds. It is not surprising, therefore, that streptococci are recoverable at times from plants grown in such fertilized soil. The noses, mouths and throats of man and animals, both wild and domestic, harbor several varieties of streptococci, sometimes as pathogens, again as saprophytes; in either case they are excreted upon the ground, clothing, bedding and other fomites. That the most pathogenic streptococci for man—group A—may live for many weeks on such fomites has been definitely established, and that animal pathogenic strains may survive on the food, bedding and stalls of domestic animals has been amply proven;



also on the hands and clothing of stablemen and milkers, as well as on milking machines and various containers used in the dairy industry. Milk is an excellent medium for growing streptococci; hence, it furnishes almost an ideal vehicle for distributing these bacteria far and wide. Special precautions are required to avoid this mode of distribution.

The milk-producing organs of animals are quite susceptible to inoculation with several species of streptococci, some of which, viz. *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis* seem to have found these organs the most favorable sites for their existence and propagation. The damage to bovine udders induced by *Str. agalactiae* causes millions of dollars annual loss to farmers. Streptococcal infections of the genital tracts of domestic animals, and fatal infections of the fetus and new born also contribute to agricultural waste. Streptococcal diseases of bees—foul brood—add their toll. Strangles in horses, induced by *Str. equi*, can ruin a cavalry troop or a racing stable. Streptococcal lymphadenitis in guinea pigs is such a serious and stubborn disease that animals among which it occurs are of little use for experiments; and often the entire stock of a grower must be destroyed in order to rid the premises of this epizootic.

How long streptococci belonging to groups other than A remain viable outside of the animal body is not definitely known. They may occur in food stuffs such as dairy products, meat or other materials wherein, at favorable temperatures, they multiply and may spoil the food, form toxic substances or induce infections. Streptococci may at times be recovered from frozen or desiccated foods long after the original processing. The presence of streptococci in soil was considered one of the sources of wound infection by these micro-organisms in soldiers installed in trenches.

All of the above-mentioned factors potentially endangering human health are of minor importance to man compared with

group A streptococcal infections. By and large, the main reservoirs of these streptococci are in human bodies, either as sites of active infection or carried on or in mucous membranes following the subsidence of such infections. In the former state they tend to be highly pathogenic, in the latter to be decreasingly so with the passage of time. Group A streptococci persistingly viable on fomites may constitute one of the important factors in the maintenance of epidemics. These streptococci may occur wherever men congregate; the more dense, intimate and prolonged this crowding, other factors being equal, the more frequently are the bacteria found. This correlation probably accounts in part for the higher incidence of streptococcal diseases among troops in barracks compared with that of armies in the field, in boarding schools compared with day schools, and in winter compared with summer environments. Streptococcal respiratory diseases are much less frequent in tropical and subtropical climates than in the temperate zone. This phenomenon is not due altogether to absence of these streptococci from the tropics, but to other as yet unknown factors. Although barrack environments were fairly uniform throughout the United States during World War II, severe streptococcal respiratory diseases were much more common in the northern camps than in the southern, and were most frequently encountered in the Rocky Mountain area. A southern locale, however, did not insure freedom from barrack epidemics when highly infectious strains of streptococci were introduced; although in these places the epidemics did not thrive with the vigor encountered in the high, dry, cold air of the mountains.

Over the past century, and particularly in the last six decades, the severity of respiratory streptococcal infections seems to have steadily decreased. This is shown statistically in the case of scarlet fever, which has become a very minor cause of death, even in the presence of a relatively per-

sistent high morbidity rate. The explanation of this phenomenon, observed in most countries, is not at hand, but the trend was unmistakable long before the introduction of modern antibiotics or even before the streptococcal etiology of scarlet fever was acknowledged. Similar phenomena have been reported by physicians in past centuries, but they lack the validity of modern vital statistics. The wide range of human diseases induced by group A streptococci is set out in another section of this chapter. With the expanding use of modern antibiotics, the opportunity for observing the evolution of these diseases is rapidly decreasing; but it must not be forgotten that at any time antibiotic resistant strains may appear and renew the multifarious picture of streptococcal infections.

## STREPTOCOCCAL DISEASES IN MAN

In man, streptococci induce many disease pictures, the relationship among which one might doubt without bacteriologic evidence. The differences stem in part from the pathogens, and in part from the patients.

### THE PATHOGENS

As already noted, some in vitro biologic functions of different streptococci are associated to a certain degree with their pathogenic capabilities. Group A strains comprise by far the majority which induce streptococcal diseases in man. "Human" group C strains also cause human diseases at times closely resembling group A infections, although usually in mild forms; and the large colony types of group G occasionally have similar disease-causing capacities. It is noteworthy that all three groups may elaborate antigenically similar streptolysin O, streptokinase, and erythrogenic toxin, also hyaluronidase. Members of other groups, which rarely possess these capacities, are nevertheless occasionally pathogenic agents in

human diseases, usually as secondary invaders of tissues already traumatized.

Diseases caused by the nonhemolytic streptococci, both viridans and enterococci, are often secondary in nature, and chronic in duration when compared with the acuteness of many group A streptococcal infections. The third general category of streptococcal infections are those induced by the strictly anaerobic strains. They are usually surgical in nature; that is, connected with wound infections, either through the skin or into a viscus such as the uterus after childbirth (Sandusky et al., 1942).

The old discussion as to whether any one specific disease is induced by any one variety of streptococcus has been answered in the negative by applying the more recently developed technics for identifying these micro-organisms: practically any disease caused by group A streptococci may be induced by representatives of several types; therefore, one may safely assert that no adjective derived from a disease can be applied specifically to any strain or strains of group A streptococci. Thus, the appellations *Str. scarlatinae* or *Str. erysipelatis* are obsolete, for in addition to many types of group A streptococci, occasionally members of groups C and G have been reported as having caused both diseases. Even the use of the adjective *pyogenes*, adopted by many bacteriologists as nosologically descriptive of group A streptococci, although useful, may lead to erroneous inferences if applied too rigidly, for some diseases caused by them are not characterized by the formation of pus. Indeed, group C streptococcal lymphadenitis of guinea pigs and strangles in horses are more characteristically purulent than is lymphadenitis of man caused by group A streptococci. The present trend in applying descriptive terms to streptococci appears to be towards adopting bacteriologically rather than pathologically derived adjectives, even though the latter long-established custom is still quite prevalent.



## STREPTOCOCCAL INFECTIONS AT VARIOUS AGES

Powers and Boisvert (1944) have emphasized the importance of age as a factor conditioning various streptococcal manifestations, to which they apply the name "streptococcosis." They furthermore describe as streptococcal fever any acute or subacute pyrexia caused by these micro-organisms.

"Streptococcal fever" in infants under 6 months of age is usually characterized by irregularly increased temperature and a moderately inflamed nasopharyngeal mucosa, with a thin mucopurulent nasal discharge which causes cutaneous excoriations about the nose. The acute episode lasts about a week, and is often followed by persistent nasal discharge and slight indisposition for about 6 weeks.

In children from 6 months to 3 years old, the clinical picture changes: the onset is insidious, and the constitutional symptoms are subacute. The fever, lasting from 4 to 8 weeks, is low grade. There is mild nasopharyngitis with a nasal discharge, usually more purulent than that in young infants. The lymph nodes draining the nasopharynx become enlarged and sometimes tender, and occasionally a single node suppurates. The inflammation may spread secondarily to the paranasal sinuses, and often to the middle ear, and produce either a simple catarrhal otitis media or a more serious purulent process. By further extension a purulent mastoiditis may result. The complexion of these children is muddy, due in part to slight anemia. The child is apt to be peevish, eats poorly, and gradually loses weight. The low-grade illness may persist for several months without its exact nature being realized unless diagnosed bacteriologically by culturing repeatedly group A streptococci from the nasopharynx. It should be emphasized that the character of "streptococcal fever" in children under three years of age is generalized in its symptomatology rather than featured by its focalization; it is subacute in contrast to the acute and stormy picture seen in later life. Furthermore, children in this age group rarely have scarlet fever or acute follicular tonsillitis. It seems not unlikely that at this period a conditioning or "retuning" of the child's tissues is occurring so that subsequently streptococcal infections assume a different character. Sequelae, such as acute hemorrhagic nephritis and rheumatic fever,

are rare in this age group, although they occasionally do occur. It is noteworthy that rheumatic fever in very young infants has been observed only in those with mothers having active rheumatic fever, which suggests a peculiar conditioning of the infant's tissues *in utero*.

The age group composed of patients from 3 to 12 years old have symptoms of an intermediate type, characterized by clear-cut nasopharyngitis and occasionally tonsillitis, and in addition, by marked general manifestations. The typical example is scarlet fever featured by a sore throat and a peculiar generalized rash. As noted elsewhere, the peculiarity of both the local pharyngeal manifestations and of the general cutaneous eruption stems from the irritating action of an erythrogenic toxin. Locally, the marked faucial edema and lingual rash, termed the enanthem, result from the oral tissues receiving a heavy dose of this toxic irritant. The scarlatinal rash is also due to this irritant, widely distributed in a somewhat diluted state through the blood stream, and not to a lodgment of streptococcal cells in the skin. This erythrogenic toxin, demonstrable in the blood during the early stages of scarlet fever, induces a rash chiefly in those individuals who react positively to the Dick test, and rarely in those who react negatively.

Scarlet fever occurs occasionally in very young children, but the incidence rises steadily from the age period of 2 to 3 years to that of 7 to 9 years, when it reaches its apex; it falls thereafter. If in this age group the individuals infected have developed sufficient antibodies to this toxin prior to the infection, they may develop an acute nasopharyngitis with marked local and general symptoms but no rash. This condition has been called *scarlatina sine eruptione*.

If such an antitoxic immunity exists in a patient having intact tonsils which receive the brunt of the attack, the resulting disease is acute follicular tonsillitis; if the tonsils have been removed, there is often an intense nasopharyngitis. It is particularly to be noted that these symptoms, indicative of the patient's ability to focalize the infections, characterize much of the "streptococcosis" of adolescence and adult life.

While these several types of infection characterize the usual modes of response in the several age periods, nevertheless in any age group there may occur very low-grade infections of the nasopharyngeal tissues

which may not be clinically manifest. The evidence for the existence of such low-grade, noncharacteristic infections may be bacteriologic or immunologic: recovery of virulent group A hemolytic streptococci from the patient's nose or throat, high or steadily increasing concentrations of antistreptolysin O and antistreptokinase in his serum. Finally, low-grade streptococcal infections may first be made clinically manifest by the appearance of acute hemorrhagic nephritis or rheumatic fever. Such sequelae begin to occur comparatively frequently in the 5- or 6-year age group, with their highest age incidence a couple of years before puberty.

The analogy between "streptococcosis" and tuberculosis consists in a progressive tendency of the inflammatory reactions to become more and more focalized and intense with increasing age of the infected individuals. The dissimilarities, on the other hand, apparently stem from the primary nature of the two infections: tuberculosis is a low-grade, chronic process in which, with recovery, the infectious agent is often confined to small foci of scarred tissue, whence relapses often originate. Many tuberculous patients are infected with only a single strain of bacilli which causes all the lesions. Some are doubtless reinfected with one or two other strains, but, if so, the second infections are often well localized and cause minor symptoms. Group A streptococcal infections, on the other hand, are usually acute or subacute in both intensity and duration; with recovery the cocci are eventually either eliminated or rendered relatively avirulent. In so far as is known, subsequent infections or superinfections are usually induced by types different from those responsible for previous infections. In other words, an individual may suffer many different streptococcal infections during his life, sometimes within one year; but rarely does he have more than one or two different tuberculous infections, and his recrudescences are often relapses of a quies-

cent tuberculosis. Doubtless each streptococcal attack "retunes" the tissues' response to subsequent attacks, but the state of resistance is less solid than in the case of tuberculosis.

#### SEPTICEMIA

The term "septicemia" comprises many conditions that characterize the entrance of bacteria into the circulation; hence, it is well to define a few terms that are specially applicable with respect to streptococci. Bacteremia designates the presence of bacteria in the blood stream. It may be temporary, as is that following tonsillectomy or tooth extraction. If the streptococci are of low virulence, few if any symptoms beyond a short pyrexia may result. If, on the other hand, they lodge in some important organ and there multiply, they may induce marked and even fatal disease. If, in addition, streptococci are continuously present in the blood, the implications are very serious. Septicemia indicates the existence of bacteremia with the production of septic symptoms either generalized or localized, usually the former. Pyemia, an older term now falling into disuse, means "pus in the blood," an exceedingly rare condition. Purulent inflammation occurring at some distance from the primary infection indicates that infected emboli lodging in these distant sites have given rise to a purulent process. The term "septicopyemia" probably better describes the disease state formerly designated pyemia.

Streptococci are the most common among micro-organisms inducing septicemia. If the streptococcal septicemia is acute, the micro-organisms are usually hemolytic and belong to group A; if chronic, they are usually nonhemolytic strains, which most commonly are thrown into the blood from a subacute bacterial endocarditis, a disease discussed later. Streptococci may enter the blood stream in one of three ways: (1) directly through a vein, a rare occurrence in the case of the hemolytic streptococci.



but probably fairly common with nonhemolytic varieties; (2) from a thrombophlebitis, contiguous to focally infected tissues, where thrombi originating in the diseased veins and containing streptococci break off and pass into the general circulation; (3) from infected lymphatic vessels, a much rarer occurrence than is the second contingency.

The signs and symptoms of septicemia comprise both general and focal phenomena. The former include high fever, often remittent in character, chills, rapid pulse rate, and marked prostration; the latter, those signs and symptoms which arise from septic emboli lodging in various organs. As septicemia is often secondary to a local infection, for example mastoiditis or endometritis, it is important to determine whether the symptoms originate entirely from the primary infection or from a complicating secondary septicemia. If the emboli arise from thrombi formed in large veins, such as those draining the mastoid or the extremities, the emboli, being large usually lodge in the lungs and give rise to symptoms and signs of pulmonary infarction. Small infected emboli, on the other hand, may be swept through the pulmonary circulation into the left side of the heart and thence distributed to various parts of the body; and in the latter contingency, many small, but generalized, embolic phenomena may occur. When the emboli originate from infected mitral or aortic valves, they lodge in the organs supplied by the greater circulation; for example, the kidneys, spleen, liver, brain and the skin. Small hemorrhages may occur also in the ocular conjunctivae or in the retina; in the latter, ophthalmoscopic demonstration is necessary. Occasionally, emboli lodging in the eyes give rise to panophthalmitis, or occluding the optic veins cause sudden blindness.

In hemolytic streptococcal septicemia there is a special tendency to involvement of joints with the induction of purulent

arthritis; aspirated, cloudy, synovial fluid contains streptococci which are usually detectable both microscopically and in cultures. This condition should be sharply differentiated from the serous polyarthritis seen in rheumatic fever, which is never purulent and occurs as a sequel of, and not during, the early acute phases of "streptococcal fever." This fluid is always sterile.

Other endothelial-lined cavities such as the pleurae, pericardium, peritoneum and meninges may also be the sites of lodgment of septic emboli which give rise to purulent pleurisy, pericarditis, peritonitis or meningitis respectively. Such purulent inflammations in large body cavities or joints are diagnosed accurately by the aspiration from them of fluid which, on microscopic examination, is shown to contain pus cells and streptococci, and from which the causative micro-organism is further identified in cultures.

#### INFECTIONS OF THE UPPER RESPIRATORY TRACT

Group A streptococci most frequently invade the human body through the upper respiratory tract from which many different diseases originate: some locally, others by contiguous spread, and still others by the transfer of the infectious agent on fingers, clothing or utensils to distant parts of the body. Dissemination via blood or lymphatic channels also occurs. Finally, toxic substances elaborated at the sites of primary infection may be generally diffused and cause symptoms.

In addition to the nasopharyngitis *per se*, which may be either acute or subacute, the infection easily travels by contiguity to the paranasal sinuses and sets up sinusitis of either short or long duration. This constitutes a special source of danger, both to the patient and to persons in contact with him, for a sinus full of pus is a dangerous reservoir for disseminating pathogenic streptococci.

Pharyngeal streptococcal infections pass readily through the eustachian tube to the middle ear and cause acute diseases, sometimes catarrhal but often purulent. If the latter occurs, the ear drum may rupture or require incision to evacuate the pus. If otitis media becomes chronic, the exudate may irritate the external auditory canal or pinna and give rise to dermatitis of these structures. More serious is extension to the internal ear and mastoid cells. With invasion of the surrounding bone, the inflammation may pass along the small blood vessels or by direct contiguity to the neighboring cerebral sinuses and induce sinus thrombosis. Portions of the infected blood clot attached to sinus walls often break off and carry streptococci to distant areas; in other words, bacteremia occurs. Again by passing directly from a diseased mastoid to the meninges, purulent meningitis may be established; or a brain abscess may result from direct extension either from the mastoid cells or meninges into the cerebral tissues.

A more remote consequence of streptococcal infection in and about the eustachian tube or of recurrent infections of the middle ear is progressive deafness. Repeated pharyngitis results in scar formation about the mouths of the eustachian tubes which become constricted; hence, equalization of the air pressure in the middle ear is prevented. Eventually, progressively increasing deafness ensues and years later becomes a distressing handicap.

In streptococcal nasopharyngitis, some tonsillar involvement usually occurs. In young children it is manifest chiefly by nonexudative enlargement of the tonsils and satellite lymph nodes. In later years, when the patient's reconditioned state tends to limit the streptococcal infection to the sites primarily invaded, the tonsils are more intensely involved in the form of acute follicular tonsillitis. These organs quickly enlarge, show a slight exudate, particularly about the mouth of the crypts, and there is

enlargement and tenderness of the satellite lymph nodes at the angles of the jaws. Some peritonsillar cellulitis may be present. At times, when this is very intense, these tissues may break down and form pockets of pus. Peritonsillar abscesses, also called quinsy, usually occur only about one tonsil. More rarely the purulent process involves the soft retropharyngeal tissues. Retropharyngeal abscesses or quinsy usually interfere seriously with swallowing and sometimes with breathing. Formerly, when streptococcal nasopharyngitis and scarlet fever were very prevalent, death from strangulation by such abscesses was not uncommon.

An even more serious condition is an intense inflammation of the tissues at the base of the tongue and the floor of the mouth. This may be in the form of a cellulitis, which at times progresses to abscess formation. This very serious condition, known clinically as Ludwig's angina, may entirely occlude the air passage and strangle the patient.

As noted elsewhere, there is a great tendency for streptococcal infections to involve various parts of the lymphatic system. Indeed, the lymphatic tissues of the oropharynx and nasopharynx, comprising Waldeyer's ring, are very frequently affected. Repeated or chronic involvement of these structures in some children eventuates in chronically enlarged adenoid tissues, which, by narrowing the posterior nasal passages, leads to mouth breathing and facial deformities. If uncorrected, this may cause upper respiratory difficulties throughout the patient's life.

The tendency for streptococcal upper respiratory infections to spread along lymphatic channels is also evidenced by involvement of the satellite lymph nodes. If the infection is chiefly tonsillar, the lymph nodes at the angles of the jaw are usually enlarged and often painful, at times very markedly so. Both from these nodes and by extension from other parts of Waldeyer's ring, the more distant cervical lymph nodes



usually become implicated. With successful localization of the streptococcal infection, the enlargement may be simple in nature, but occasionally the lymph nodes suppurate and the abscesses break through the cervical integument. Occasionally, the pus burrows downward into the mediastinum, and sometimes bacteremia arises from suppurated lymph nodes.

In scarlet fever, and sometimes in severe tonsillitis, a general lymph adenopathy occurs, probably from irritation by a soluble toxin. With recovery, this lymph node enlargement recedes, but sequelae, such as hemorrhagic nephritis or rheumatic fever, are often heralded by renewed enlargement of the cervical lymph nodes and sometimes by a relapsing general adenopathy. In fact, recurring adenopathy is probably the most common of such sequelae and may occur without clinical evidence of more serious visceral diseases. Careful documentation of the condition of the lymph nodes, both during and following upper respiratory streptococcal infection, is therefore an important duty of the physician.

#### INFECTIONS OF THE LOWER RESPIRATORY TRACT

This area may be infected with group A streptococci either by extension downward of a nasopharyngitis or by aspirating bacteria directly into the lungs. The former is probably the more frequent: laryngitis, tracheitis, bronchitis and bronchopneumonia occur in rapid succession. The latter, accurately described as interstitial pneumonia (MacCallum, 1919), is characterized by marked acute inflammatory reactions in and about the bronchi. These become plugged with fibrinopurulent exudate and desquamated epithelium; and areas distal to the occluded bronchi may collapse. The peribronchial exudate is quickly invaded by histiocytes and tends to organize. The finer blood vessels are often filled with fibrin thrombi. Even more striking is in-

volvement of the lymphatic vessels, where characteristic streptococcal lymphangitis occurs. Streptococci invade a lymphatic vessel; wandering leukocytes accumulate in the lymph; the bacteria are swept forward into the peribronchial lymph nodes where they are arrested and set up purulent lymphadenitis. If the lymphatic vessel becomes occluded, the infection spreads in a retrograde direction towards the surface of the lung; an area of focal pleurisy is induced, followed quickly by an outpouring of serofibrinous exudate, first quite fluid, but shortly it becomes loaded with streptococci and leukocytes. Such large amounts of fluid accumulate in the pleural cavity that the lung may be compressed to a fraction of its normal size. The tendency for the fibrin covering the lung to organize makes expansion of this organ difficult or impossible. Areas of lobular pneumonia may be interspersed between those of interstitial pneumonia, and occasionally these break down and form abscesses. Needless to say, this is a serious and often fatal disease because of intense toxemia, septicemia and involvement of so many intrathoracic structures. Pericarditis is not infrequent.

During World War I this type of streptococcal pneumonia occurred among thousands of trainees, first as a complication of measles, then of scarlet fever, and eventually as a primary condition. Later similar pneumonitis was seen in many fatal cases of influenza.

Secondary streptococcal infections may indeed be serious complicating factors in primary virus-induced respiratory diseases. With the aid of modern antibiotics, these streptococcal complications can usually be controlled; hence will probably be infrequent in the future unless antibiotic-resistant strains become widely distributed.

Among pulmonary diseases that may be attributed to streptococci is the peculiar form of pneumonia encountered in rheumatic fever, because the latter is a sequel of group A streptococcal infections. In con-

tradistinction to the usual occurrence of streptococci in the inflamed areas and exudates in primary streptococcal pneumonia, these micro-organisms are rarely, if ever, encountered in the rheumatic lung. The blood vessels appear to be primarily involved, with fibrin thrombi in the inter-alveolar capillaries, and varying degrees of inflammation of the medium-sized arteries and arterioles. Periarteritis is common. Early there is a pouring out of leukocytes and fibrin into the alveoli; later a proliferation of histiocytes, combined with a cuboid thickening of the epithelial cells lining the alveoli. In the chronic form, granulomalike areas are sometimes encountered.

Nonhemolytic *Str. MG*, described by Mirick et al. (1944), apparently plays some unexplained role in the pathogenesis of atypical pneumonia in about 50 per cent of the patients (Horsfall, 1947). It has been recovered from the lungs in several fatal cases and from the sputum of about two-thirds of the patients with this disease; also much less frequently from the sputum of patients with other respiratory infections, and occasionally from normal people. Early in atypical pneumonia no antibodies against this streptococcus are found in patients' sera, but about half of them develop antibodies between the second and fifth week. Positive skin reactions may also be elicited at this time with the soluble specific polysaccharide which these streptococci elaborate. While *Str. MG* has not been shown to be pathogenic for laboratory animals, with the exception of the chick embryo, nevertheless, the phenomena above detailed suggest that this micro-organism may play an accessory role in atypical pneumonia.

#### INFECTIONS OF THE SKIN

Cutaneous streptococcal diseases offer unusual opportunities for observing the multi-form responses to infection with these micro-organisms. It is hard to imagine how such diverse clinical pictures as impetigo

contagiosa, erysipelas, aural eczema from otitis media, the rash of scarlet fever, streaking lymphangitis from a puncture wound, and the erythema marginatum of rheumatic fever could all stem from group A streptococcal infections, and yet this seems to be the case. Doubtless, these manifestations are conditioned by several factors: the age of the individual; his previous streptococcal infections; and the capacity of the infecting strains to elaborate certain pathogenic substances in various concentrations, together with the patient's resistance or susceptibility to these substances.

While formerly it was thought that most cutaneous streptococcal infections arose primarily in the skin, our ability to identify strains immunologically has demonstrated that often an apparently primary cutaneous disease is in fact secondary to a streptococcal infection elsewhere, often in the nasopharynx. At times streptococcal dermatitis is secondary to other diseases; for example, herpes simplex lesions, virus induced, may become secondarily infected with streptococci and quickly lose their unique characteristics. Similarly, secondary streptococcal infections may complicate dermatophytosis in adults or atopic eczema in infants. In such infected infantile eczema, the secondary involvement is often characterized by extensive and markedly indurated erythema and a serosanguinous exudate; at times there is combined crusting and weeping of the surface, a most important diagnostic feature. Satellite adenopathy is quite marked in these streptococcal eczemas. In two-thirds of the cases of such infantile eczema, Boisvert and Powers (1944) recovered homologous types of group A streptococci from the cutaneous and nasopharyngeal lesions of the respective patients; occasionally, group G streptococci occurred in both skin and nose. The primary lesion was apparently upper respiratory, whence the streptococci were transported on the skin of the fingers of the child or attendant.



Curing the primary nasal infection is often requisite to healing the dermatitis.

Intertrigo is an erythematous and often weeping lesion of the skin in areas where two-folded layers are in constant contact. Encountered in both children and adults, it is often apparently caused by hemolytic streptococci.

Impetigo contagiosa is a disease with marked cutaneous alteration and very little general bodily disturbance. It is apparently due to a primary invasion of the most superficial cutaneous layers by streptococci which seem to elaborate a lytic agent that dissolves the intercellular cement of basal epithelial cells. There is a rapid accumulation of clear, watery, serous fluid containing a few leukocytes, which forms flat blisters varying in diameter from a few millimeters to 2 or 3 centimeters. From the clear fluid aspirated early, pure cultures of hemolytic streptococci can usually be grown. The lesion soon becomes secondarily infected with staphylococci normally residing in the skin. The blister's surface becomes covered with a brownish, so-called "candy crust." The serous exudate infects other cutaneous areas where new blisters form; these in turn infect still other areas and successive repetition of the process may implicate much of the skin. Other persons who are easily infected with the exudate show similar pictures. While mixed infections practically always characterize this disease, streptococci seem to play the predominant primary etiologic role. There is a minimal involvement of the subepithelial tissues. When properly treated, the very superficial lesions heal quickly and leave few residua and little obvious scarring. Impetigo is most common in children, which possibly accounts, in part, for the superficial nature of the tissue changes; for young children, as a rule, have had little previous experience with streptococci to retune their tissues to streptococcal infections. The fact that adults may also contract impetigo in-

dicates that other elements are operative in its pathogenesis.

Erysipelas (St. Anthony's fire), a unique clinical picture recognized for over two millennia, is caused by hemolytic streptococci, usually members of group A but rarely of group C. It is characterized by a red, brawny, cutaneous thickening, which begins as a minute lesion and spreads marginally for four to six days. The red margins on palpation present a peculiar hard indurated "wall"; but all the diseased skin has a rubbery or tallowlike consistence. On the face, erysipelas, usually beginning near the external nares, often spreads over both cheeks in a butterflylike pattern; but the margins rarely extend into the hair. With the development of an effective general resistance, fever and toxic symptoms subside, local extension stops and recovery begins. If the eyelids are involved, the edema may seal them shut, and even invade the peri-orbital tissues. In other areas the lesion is round or irregularly oval. Occasionally on the surface there are many small blisters filled with fluid from which streptococci may be grown; and rarely the indurated skin softens focally and forms abscesses. Histologically, the inflammation involves mainly the superficial lymphatic vessels which are crammed with fibrin, leukocytes and chains of streptococci. There is also edema of the perilymphatic tissues. The peculiar evolution is due to the progressively diffuse lymphatic vessel involvement.

Signs and symptoms of general intoxication and fever accompany the local lesion. These continue as long as the erysipelas spreads; shortly after it stops, the general signs disappear. Probably the streptococci always gain entrance into the skin through some abrasion, although this initial site is usually not demonstrable. When the cutaneous lesions first appear near the external nares, there probably was a prior streptococcal infection in the nose or sinuses, whence the micro-organisms were carried to the skin. Carefully correlated clinical and

bacteriologic data frequently indicate such a sequence.

Erysipelas was formerly one of the most serious complications of wounds, whether inflicted traumatically or surgically, and before Pasteur and Lister it was the surgeon's *bête noire*. Fortunate indeed was the post-operative patient who escaped its ravages. It is most often fatal in the aged and in young infants. Its occurrence in very young infants indicates that the peculiar manifestations are not necessarily due to a reconditioning of the body from previous streptococcal infections; at least it can apparently occur with primary streptococcal infections or shortly after such infections of the upper respiratory tract. The complete clinical picture detailed above rarely occurs today because adequate antibiotic therapy quickly arrests its progress.

In addition to the foregoing rather characteristic skin diseases due to streptococci, these micro-organisms cause other pathologic pictures in this tissue. The simplest, a puncture wound which introduces a few highly virulent streptococci into the subcutaneous tissue, may be only a small inflamed area which is followed shortly by a red streak running towards the satellite lymph nodes. This is evidence of subcutaneous lymphangitis; and the redness is probably due to a poison elaborated by the streptococci. The first lymph nodes involved quickly become enlarged and tender. If not arrested here, the infection passes along lymphatic vessels to the next lymph nodes. From any infected node a bacteremia may be established. The infected puncture wound may quickly develop into a localized cellulitis which may then suppurate. Again, although remaining quite small, the inflammation may involve contiguous blood vessels, and the resulting thrombosis may compromise the nutrition of the tissues distal to this point and thus cause localized gangrene. The dead tissue becomes a *locus minoris resistentiae* in which streptococci easily pro-

liferate and this constitutes an additional source of danger.

Cutaneous burns, if of any extent, form fertile fields for hemolytic streptococci to proliferate and set up a diffuse inflammation, with marked weeping edema, purulent exudate, and severe general symptoms due to toxic substances absorbed from the infected burns.

Recurrent tropical lymphangitis is characterized by repeated acute attacks of lymphangitis, usually in the skin of the legs, with enlargement of the satellite lymph nodes and signs of acute intoxication. The streptococci gain entrance to the tissues through fungous lesions or other primary injuries. Each attack increases the thickness of the skin. Often chronic ulcers persist in these thickened areas and become sites of subsequent streptococcal infections, usually by members of group A, sometimes of groups C and G. The repeated attacks eventually cause the permanent cutaneous thickening designated elephantiasis. This suggests that much tropical elephantiasis is due to recurring streptococcal lymphangitis and not necessarily to filariasis.

Besides those dermatologic maladies in which streptococci are recoverable directly from the lesions, there are dermatoses included under the rubric *erythema multiforme*. In those instances due to streptococci, the micro-organisms are probably focalized elsewhere and the dermatosis is apparently due to some toxic substance. These lesions rarely suppurate. A peculiar form of erythema, designated *marginatum*, spreads with a sharp red marginate border beginning as rings, which merge, then break into segments leaving irregular edges and clear centers. Although quite characteristic of rheumatic fever, it is encountered in patients who show no other signs of this disease.

#### SCARLET FEVER

Scarlet fever, aside from erysipelas the most sharply defined disease induced by



hemolytic streptococci, was formerly a commonly fatal infection. Its etiology was undetermined until the early 1920's, but due to its characteristic clinical and epidemiologic aspects, it became reportable early in the nineteenth century; hence it is possible to trace its changing manifestations for nearly a century and a half. Today, although having a high morbidity rate, scarlet fever is almost universally a relatively benign infection. Prior to World War II, practically the only European areas where there persisted the malignant forms, so common a century earlier, were in Roumania and the neighboring countries. Today the disease is important not as a rapid killer but because of its remote crippling sequelae.

Scarlet fever may be considered as a nasopharyngitis with a skin rash in a person infected with streptococci which produce an erythrogenic toxin, against which the patient has no effective antitoxic immunity. The erythrogenic toxin poisons the vascular endothelial lining, the parenchymal cells of the viscera, such as the liver and kidney, occasionally the myocardium. This toxin, produced in the nasopharyngeal tissues, circulates in the blood, occasionally in concentrations as high as 300 units per cc., but usually in about one-tenth this strength. It usually is present in the blood concurrently with the manifestations of active intoxication, i.e., for three to seven days, and parallel with its disappearance from the blood, the general signs and symptoms ameliorate. It is also recoverable in the urine of many patients after a lag of a few days. The toxin occurring in the highest concentration in the throat induces there intense diffuse redness and some edema, and in the tongue a swelling of the papillae resulting in the so-called "strawberry tongue."

The characteristic exanthem involves particularly the softer skin on the flexor aspects of the arms and on the axillary, inguinal and anterolateral surfaces of the trunk; at times there is involvement of the entire skin with the exception of that of the face,

which shows merely a febrile flush contrasting with a very characteristic circumoral pallor. The typical scarlatinal rash combines a diffuse bright redness with darker punctate erythema. Pressure causes blanching which slowly disappears following its removal; first the tiny deeper red punctate spots reappear, followed by the diffuse flush. That the erythema results from an irritation of the vascular tissues by the erythrogenic toxin can be demonstrated by the Schultz-Charlton extinction phenomenon: intracutaneous injection of antitoxic sera causes the rash to disappear locally. This phenomenon is demonstrable during the first three or four days of the disease, but is less marked later when the toxin is thoroughly fixed in the cells and relatively non-neutralizable. In very toxic cases, cutaneous hemorrhages indicate that an unusually potent toxin has destroyed the vascular lining. The rash usually begins to disappear about the end of the first week of the disease; then desquamation of the involved skin begins. Scaling over the trunk, arms and legs is fine and branlike, while over the palms of the hands and soles of the feet the desquamated skin may peel off in casts. Formerly the desquamations were thought to contain the infectious material; hence, the period of isolation corresponded with that of peeling. This fallacious idea has been discarded.

Most cases of scarlet fever are accompanied by signs and symptoms of general intoxication which vary in intensity and disappear with the development of antitoxic immunity. The toxic form of the disease is characterized by early intense intoxication, marked prostration, high fever, tachycardia, and sometimes by cutaneous hemorrhagic areas, jaundice and eventual heart failure. Adequate doses of antitoxic sera neutralize such manifestations.

The septic forms of scarlet fever show signs of distribution of the streptococci to the skin and viscera remote from the nasopharynx. The local complications comprise a spreading of the infection into the para-

nasal sinuses, middle ear, mastoid, and contiguous structures. Occasionally there appear peritonsillar or retropharyngeal abscesses, and at times Ludwig's angina. The cervical lymph nodes are practically always enlarged, and occasionally suppurate. Septic complications usually respond to adequate antibiotic therapy, although abscesses require surgical attention. They are largely prevented by early and continuous penicillin treatment.

The sequelae of scarlet fever, hemorrhagic nephritis and rheumatic fever, remain the most serious features of the disease. They are later discussed more fully, for they are of the same nature as those which follow other group A streptococcal infections. Here, however, it is well to emphasize the importance of being on the alert for recurring lymphadenitis and the appearance of bloody urine, facial edema, polyarthritides, or carditis in the weeks following an attack of scarlet fever. With the current custom of releasing these patients from quarantine in the second or third week of the disease, there are lessened opportunities for detecting sequelae, particularly those in the subclinical zones, and in many patients postscarlatinal carditis occurs but remains undetected until later cardiac disabilities appear.

#### PUERPERAL FEVER

Puerperal fever, formerly very prevalent, has fortunately become a relatively rare condition. It is a special form of wound infection. Following childbirth the female genital tract presents several kinds of open wounds: endometrial areas from which the placenta has been stripped, cervical lacerations, and perineal tears. All are especially susceptible to infection. While the normal vagina is often inhabited by many varieties of streptococci, the strictly anaerobic forms have the most serious implications with respect to puerperal fever. The microorganisms responsible for this condition are usually introduced from the following more re-

mote sources: (1) from the patient's own nasopharynx, when it is the site of an acute or subacute streptococcal inflammation or of carriage of virulent streptococci; (2) from other patients having streptococcal diseases or wound infections; (3) from the nasopharynges of attending physicians or nurses; (4) from fomites, such as instruments or clothing connected with patients who are eliminating streptococci, or from dust and lint from the bedding of such patients. Recognizing these several sources of infection and excluding them from parturient women has resulted in the markedly decreased incidence of puerperal fever.

Postpartum sepsis may be limited to an endometritis, a local infection of the uterine lining with microorganisms of low virulence, usually not belonging to group A, and which invade retained placental fragments or blood clots. The inflammation does not extend through the uterus into the broad ligaments or neighboring veins; hence there is slight danger of infected emboli being cast into the blood stream. The clinical picture is one of local inflammation with generalized symptoms of toxemia. Surgical removal of the infected placental residua and blood clots usually induces recovery.

If, however, the condition is due to virulent group A streptococci, one or two serious and often fatal complications quickly ensue: septicemia, with symptoms described under that heading, and/or peritonitis, the result of extension of the inflammatory process from the uterus into the broad ligaments, thence to the neighboring peritoneum, whence it may involve the entire peritoneum. Septicemia may originate from thrombosis of the uterine veins. Growing thrombi extend into the internal iliac vein, break off, and lodge in the lungs where they give rise to purulent infarcts. Lymphatic vessels comprise another channel for dissemination of puerperal infections. Cellulitis of the broad ligaments usually stems from infected uterine, cervical or vaginal lacerations; while blood stream distribu-



tion of streptococci sequential to thrombophlebitis usually indicates a panmetritis. Occasionally septicemia follows direct bacteremic invasion through blood vessel walls without the occurrence of either thrombosis or lymphangitis.

In uncomplicated hemolytic streptococcal infections of the female genital tract, the lochia are usually not foul. Foulness indicates either a mixed infection or one due to the anaerobic *Str. putridus*.

Occasionally puerperal scarlet fever is seen. The genital tract is infected postpartum with streptococci elaborating erythrogenic toxin to which the woman is not immune. The symptomatology is that of scarlet fever without nasopharyngitis but with metritis. Rarely erysipelas originates from hemolytic streptococcal genital tract infections.

Streptococci not infrequently infect the urinary tract. While the responsible microorganisms occasionally belong to group A, they are more frequently members of groups D or B or viridans streptococci, that is, streptococci normally encountered in the intestines. When caused by enterococci, urinary tract infections are quite resistant to sulfonamides and penicillin, and even though held in check by these drugs, recurrences often follow their withdrawal. Cystitis, ureteritis, pyelitis, and pyelonephritis may be caused either by streptococci becoming implanted primarily in the bladder and then spreading upwards towards the kidney, or occasionally by the bacteria reaching the kidney through the blood stream. Following acute gonococcal prostatitis, a chronic inflammation is sometimes introduced by streptococci which have become implanted secondarily in the prostate.

#### ENDOCARDITIS

Endocarditis illustrates strikingly the numerous clinical pictures that may emerge from infection of one tissue, the endocardium, with streptococci having markedly

different biologic properties. These range from highly virulent group A strains to lowly virulent nonhemolytic varieties and representatives of other groups.

Acute septic endocarditis, acute malignant endocarditis, or acute ulcerative endocarditis, also induced by other bacteria, is mostly caused by group A streptococci which become implanted on the heart valves, generally the mitral and aortic. There they establish an infected area whence the microorganisms, either free or in small emboli, are widely disseminated. Most commonly the lesions are polypoid, and composed mainly of components derived from the blood. Sometimes ulceration of the valves occurs with loss of substance; such ulcers are covered by thin layers of exudate. Occasionally they perforate a cusp. More rarely, small gray or reddish verrucae form on the valves. This disease in brief is an acute infection localized first on the endocardium with secondary acute general manifestations of septicemia similar to those occasioned by primary streptococcal diseases elsewhere. The special features originate from the endocardial lesions which, during life, are sometimes disclosed by murmurs indicating disturbed valvular functions. Often, however, there may be no organic murmurs to suggest such a localization, and the endocarditis may be revealed only postmortem.

Subacute bacterial endocarditis, or *endocarditis lenta*, as its name indicates, is characterized by a subacute or chronic course and by a peculiar endocardial inflammation. Although induced by many different lowly virulent microorganisms, it usually is caused by nonhemolytic streptococci, mostly by those which produce green colonies on blood agar. Some of the varieties of nonhemolytic streptococci are discussed elsewhere. Recently, one having marked penicillin resistance and designated as *Str. SBE* or *Str. sanguis*, has been recovered from about one-third of these patients.

About one-third of the cases are induced

by *Str. salivarius*, a common throat viridans streptococcus. Other streptococci having their habitat in the throat or gastro-intestinal tract such as *Str. mitis*, *Str. bovis*, enterococci, group G streptococci, and occasionally group B streptococci, and more rarely members of other groups have also been recovered from patients with subacute bacterial endocarditis. Probably these lowly virulent streptococci frequently enter the blood stream from traumatized areas of the mouth and intestinal tract, and occasionally from the genito-urinary tract, especially postoperatively, but such transient bacteremia is relatively innocuous unless the bacteria lodge on the endocardium and there proliferate. This practically always occurs on previously diseased valves, usually those scarred by rheumatic fever and less frequently on those congenitally malformed. Occasionally, the streptococci lodge in an arteriovenous aneurism. These lesions generally involve the left side of the heart and are situated both on the valves and chordae tendineae, occasionally on mural endocardium. They consist of flat, cauliflowerlike excrescences containing fibrin, platelets, and other inflammatory products in which the streptococci are embedded. This embedment makes it difficult to attack them with therapeutic agents.

Clinically, the disease follows a subacute or chronic course. Often it is revealed only by low-grade fever and toxemia with gradually increasing anemia; and not until emboli break from the lesions and lodge in various parts of the body are the characteristic clinical pictures of the disease made evident. These embolic manifestations, in contrast to those of acute hemolytic streptococcal endocarditis, are not characterized by intense inflammatory reaction but by relatively bland infarcts of varying size, dependent upon the diameter of the occluded blood vessel. Serious symptoms arise from disturbed function of the particular organs in which infarcts occur. The spleen in contradistinction to the soft organ of

acute sepsis is practically always large and hard, both from hyperplastic splenic tissues and from infarction. In the kidneys there occur peculiar glomerular lesions and occasionally wedge-shaped infarcts. When small emboli plug *vasa vasora* of large arteries, embolic aneurisms occur at the sites of lodgment. In the skin, mucous membranes and conjunctivae, characteristic petechiae often appear in showers. They may be embolic phenomena, but cutaneous petechiae also result from the rupture of fragile vessels. While painful periartritic areas are fairly common, exudative arthritis is very rare. Serofibrinous exudates into the various large body cavities are also uncommon, even though many petechiae may occur on the pleurae, pericardium or peritoneum.

The diagnosis rests upon repeated demonstration of characteristic streptococci in blood cultures, upon the appearance of the peculiar embolic phenomena together with the physical signs of endocarditis. This disease was formerly almost invariably fatal, but lately intensive and prolonged penicillin therapy has materially changed the outlook; even with recovery, however, distorted valves remain to plague the patient.

#### SEQUELAE OF GROUP A STREPTOCOCCAL INFECTIONS (ACUTE NEPHRITIS, RHEUMATIC FEVER AND ERYTHEMA NODOSUM)

The manifestations of acute streptococcal diseases are divisible into three general classes: (1) primary, due to the immediate infection; (2) complications, usually beginning during the second week in an organ other than that originally affected, caused either by the original infecting streptococcus or by superinfection with heterologous strains; (3) sequelae, in which the manifestations apparently do not stem from streptococci implanted in the diseased tissues but from alterations in those organs which are possibly induced by soluble prod-



ucts from the micro-organisms in areas primarily affected.

That important alterations occur in tissues distant from those originally infected is shown by recording frequently the state of all superficial lymph nodes of a patient for several weeks following a streptococcal infection. The satellite nodes draining the area primarily infected are usually enlarged, and a general adenopathy often occurs in scarlet fever and tonsillitis. With recovery the lymph nodes diminish in size; not infrequently a secondary enlargement of the satellite nodes occurs, and at times, during the third to sixth week, there is a renewed enlargement of all the lymph nodes (Escherich and Schick, 1912). Patients recovering from tonsillitis occasionally have in the third or fourth week a recurring non-exudative tonsillar enlargement with a diffuse redness of the neighboring pharyngeal wall, but no bacteriologic evidence of reinfection. Likewise, weekly or biweekly urine examination for a month or six weeks following a hemolytic streptococcal infection often shows, after the disappearance of the initial febrile albuminuria, a later recurring albuminuria and an increase in cells, as revealed by an Addis count. These abnormalities indicate a late temporarily altered renal parenchyma, not sufficiently intense to be designated nephritis.

Streptococcal infections with sequelae may thus be divided into three phases (Schick): (1) the acute primary infection; (2) a quiescent or latent period; (3) nephritis or rheumatic fever.

In acute hemorrhagic nephritis which follows hemolytic streptococcal infections, we may picture the slight pathologic state described above as progressing to severe renal damage, indicated by bloody urine, marked albuminuria, many granular casts, and distinctly disturbed renal function. Azotemia may ensue with intense general intoxication, edema of the face, and occasionally of the body. Unless such severe nephritis quickly regresses, death ensues.

Usually, however, the patients recover, and, in contrast to rheumatic fever, there is peculiarly little tendency for hemorrhagic nephritis to recur following a subsequent streptococcal infection. While acute hemorrhagic nephritis is not always preceded by a hemolytic streptococcal infection, nevertheless, scarlet fever, tonsillitis or streptococcal nasopharyngitis are usually precursors of this condition, which occurs most commonly in children and adolescents. At times the streptococcal infection of the nasopharynx or sinuses is so slight that it is missed clinically and only detected bacteriologically.

Of all the sequelae of hemolytic streptococcal diseases, rheumatic fever is the most serious because it commonly eventuates in chronic cardiac valvular disease. While discussion and doubt concerning the etiology of rheumatic fever still prevail, and although its exact pathogenesis remains unexplained, nevertheless, utilization of modern bacteriologic methods has established the fact that the acute disease is practically always preceded by group A hemolytic streptococcal infections. These may be relatively mild, even subclinical, or severe and easily diagnosed, for example scarlet fever. Indeed, postscarlatinal rheumatism was long thought to be a special manifestation of that disease; but since its streptococcal etiology has been established, it appears obvious that the rheumatic fever following all streptococcal infections is similar in nature etiologically, clinically, pathologically, and in the ultimate cardiac consequences (Swift, 1947). As with acute hemorrhagic nephritis, moreover, the precursory streptococcal infections may be so subclinical that their presence is demonstrable only bacteriologically or by application of serologic technics, which show increased antibodies against antigenic components of group A streptococci. Such immunologic evidence is often required, because in about one-third of the patients with rheumatic fever, the hemolytic streptococci have disappeared

from the nasopharynx when the rheumatic symptoms appear.

Rheumatic fever is rare in infancy, but begins to occur in children about 3 years of age. Thereafter its relative incidence steadily increases for 7 to 8 years, then drops; but many first attacks occur in youth or early adult life, and may even be encountered in the fifth or sixth decades. There is, however, a decreasing incidence of all streptococcal diseases in the later age groups, although they are encountered in the aged. Probably between 5 and 20 per cent of acute upper respiratory hemolytic streptococcal infections are followed by rheumatic fever.

This disease sometimes begins in the second week following an acute streptococcal infection, but more frequently it occurs in the third week, and its onset may even be delayed five or six weeks. The typical attack begins acutely with signs of general intoxication, sometimes a renewed sore throat, and is characterized by a migratory, nonpurulent polyarthritis, and sometimes by polyserositis with a serofibrinous bacteria-free exudate. More important still is cardiac inflammation which may occur in any or all parts of the heart: endocardium, myocardium or pericardium. Often there is a concomitant vasculitis; hence, the carditis may be regarded as only a special example of a general vascular system disease.

Rheumatic valvulitis, a nonpurulent, interstitial inflammation of the valve cusps and rings, is particularly characterized by the formation of tiny warty verrucae along the valve margins; they are characteristic but not pathognomonic of rheumatic fever. With healing, scars replace the verrucae and also occur in the valve cusps and chordae tendineae. Thickened scars eventually distort the valves, and this disturbs seriously the cardiac function. Rheumatic myocarditis, often present, gives a characteristic picture of tiny disseminated perivascular granulomata—the Aschoff bodies.

A peculiarly serious characteristic of rheumatic fever is its tendency to recur following subsequent hemolytic streptococcal infections. Such recurrences often lead to renewed carditis with increased valvular damage and decreased cardiac function. Some patients suffer many recurrences, each one probably due to a new streptococcal infection. Most patients tolerate fairly well the damage inflicted by one or two rheumatic attacks, but many repeated attacks, each increasing the cardiac damage, compromise progressively the patient's strength and shorten his life; hence, from a public health aspect, the most important measure aimed towards preventing progressive rheumatic heart disease is the protection of such patients from repeated hemolytic streptococcal infections. While about 10 per cent of active rheumatic fever patients show evidence of slight renal disturbance, only an occasional one develops clear-cut hemorrhagic nephritis.

A rheumatic fever patient may have polyarthritis and little evidence of carditis or vice versa. There may be neither polyarthritis nor polyserositis, and even little fever to indicate the existence of the disease. In fact, detectable manifestations may be limited to the heart, and be only demonstrable electrocardiographically or stethoscopically. The frequency of nonsymptomatic poststreptococcal rheumatic carditis is being increasingly recognized. Its existence explains the denial by many patients of having had symptoms referable to rheumatic fever, even though they have advanced rheumatic heart disease.

The intimate association of rheumatic fever and chronic carditis with group A streptococcal infections makes advisable the periodic examination of every patient with acute streptococcal infections for 4 to 6 weeks for the signs of these sequelae. Because they are usually accompanied by abnormal erythrocyte sedimentation rates, either continuing from the primary streptococcal infection or recurring after having



become normal, it is advisable to measure the ESR every 7 to 10 days and to examine further those with abnormal rates for evidence of nephritis or rheumatic fever, which may profoundly influence their later life.

While from a public health standpoint streptococcal diseases appear less menacing because puerperal fever, septic sore throat, severe scarlet fever, and erysipelas have universally decreased, even before modern antibiotics were available, still the yearly occurrence of many thousands of cases of rheumatic heart disease and the continuing high annual death rate from this disease indicates its persisting importance. Moreover, even though sulfonamides or penicillin may control the acute manifestations of streptococcal disease, nevertheless, many patients receiving therapeutically adequate doses during the first two weeks of a streptococcal disease still develop acute hemorrhagic nephritis or rheumatic fever. There are data suggesting that very intensive penicillin treatment begun the first or second day of streptococcal nasopharyngitis and continued two or more weeks may prevent the appearance of sequelae. The chief demonstrable value of antibiotics with respect to rheumatic fever is their ability to prevent the streptococcal infections whence stems rheumatic fever.

Erythema nodosum sometimes follows hemolytic streptococcal infections, although it also occurs as a manifestation of tuberculosis. In both diseases the characteristic nodular redness has been induced by intracutaneous injection of extracts prepared from the respective bacteria, but streptococcal extracts have not induced them in tuberculous subjects or vice versa.

Erythema nodosum is characterized by the appearance on the extremities, usually their extensor surfaces, of successive crops of small to medium-sized red spots which become nodular, and very painful. In receding, each lesion changes from red to purple, then to brown and fading yellow, when the areas resemble nonindurated bruises. Fre-

quently, fever, general intoxication, and sometimes mild arthritis accompany the rash. While some observers consider it a complication of rheumatic fever, erythema nodosum occurs in many patients without accompanying arthritis or carditis. Indeed, chronic cardiac valvular disease is not a common late sequel. There are data suggesting that successful cure of streptococcal infections accompanying erythema nodosum also causes the skin lesions to recede, but because they usually resolve spontaneously, it is difficult to evaluate the therapeutic significance of this phenomenon.

#### ANAEROBIC STREPTOCOCCAL INFECTIONS

Anaerobic streptococci comprise a special category of Gram-positive, nonhemolytic, chain-forming cocci, which require for their growth either strict anaerobic conditions or only minute quantities of free oxygen. They are recoverable from some apparently normal human mouths, intestines, and vaginae, where they live apparently as harmless saprophytes or commensals. In fact, among the many normally existing vaginal streptococci they have the highest pathogenic potentialities. In mechanically traumatized tissues or as secondary invaders of lesions infected primarily by other micro-organisms they may give rise to suppuration (Sandusky et al., 1942).

Occasionally they cause a special form of postpartum sepsis by spreading from the vagina to the uterine cavity, where they induce endometritis in which the lochia have a peculiar, foul odor. If secondary pulmonary abscesses occur, the patient's breath emits a similar smell. Such postpartum emanations should suggest this condition and bacteriologic examinations specially calculated to identify these bacteria. In addition to their anaerobic requirements, they produce in media a gas with a putrid odor, the result of  $\text{SO}_2$  formation.

They have also been recovered in non-obstetric genital-tract infections, from non-

puerperal pulmonary abscesses and from purulent mastoiditis; in the latter cases they probably traveled from the patient's mouth. They sometimes induce lesions near the

lower intestine such as perirectal abscesses, and infected pilonidal cysts; and occasionally hepatic abscesses contain them in pure culture. They also occur on the human skin

TABLE 30. COMPARATIVE PATHOGENICITY OF SEROLOGIC GROUPS OF HEMOLYTIC STREPTOCOCCI

ANIMAL	CHIEF DISEASES		INFREQUENT DISEASES		FOUND AS CAR- RIAGE STRAINS OR PATHOGENICITY UNDETERMINED
	STREPTOCOCCUS GROUP	DISEASE	GROUP	DISEASE	
Man	A	Many (see text) (90-95 per cent)	B, C, D, F, G	See text	A, B, C, D, F, G, H, K, L, M
Cattle	B: <i>Str. agalactiae</i>	Mastitis (80-90 per cent)	A	Mastitis, rare: dangerous for man	B, C "animal," D, E, G, H, K, L
	C: <i>Str. dysgalactiae</i>	Mastitis (5-10 per cent)	B	Abortion	
	— <i>Str. uberis</i>	Mastitis (5-10 per cent)	C "animal"	Metritis, abortion, septicemia, arthritis (calves), erysipelas, wound infections	
Horse			G	Mastitis, rare	
	C: <i>Str. equi</i>	Strangles	A	Metritis, abortion	C "animal,"
	C: "animal"	Respiratory and wound infections	B	Metritis, abortion	C "human," D
Dog		Wound infections	C "animal"	Metritis, abortion, arthritis (foal), respiratory infections	
	C: "human"				
Monkey	G: large colony	Genital tract infections	G	Respiratory infections	A, B, C, D, E, G, J, M
			L	Birth complications, genital tract infections	
			M	Respiratory infections	
Sheep					
Guinea pig			A	Skin infections, "erysipelas," septicemia	A, C "human," G
			C "human" (?)		
			G	Respiratory infections	
Swine					
Chickens					
Goats					
Rabbit					
Mouse					
Fox					
Ferret					
Bees					



and in wounds involving this structure, and in bruised or crushed tissues they find conditions specially favorable for growth.

As a general proposition, wound infections or nontraumatic abscesses containing malodorous pus indicate special efforts for identifying anaerobic streptococci bacteriologically, because no other streptococci give rise to similar foul discharges. Not all anaerobic streptococci, however, produce fetid or putrid odors. If, therefore, streptococci are found microscopically but are not recovered bacteriologically with aerobic technics, then one should resort to anaerobic cultures, which, indeed, some bacteriologists recommend as routine procedures.

Attempts to relate these streptococci immunologically to streptococcal groups A to N or to the various aerobic nonhemolytic or viridans streptococci have, so far, failed. Indeed, no adequate system for classifying these strictly anaerobic streptococci has been devised. Although the usual anaerobic, fetid odor-producing streptococci are non-hemolytic on blood agar, and often produce a green circumcolonial color, there are apparently several other varieties of anaerobic streptococci with different physiologic capacities. Some are hemolytic. The common factor among these streptococci is their requirement of an anaerobic environment for growth. They have sufficient pathogenic potentialities to indicate applying anaerobic cultural technics in most cases of doubtful diagnosis.

#### STREPTOCOCCAL DISEASES OF LOWER ANIMALS

(See Table 30)

Domestic and wild animals suffer from streptococcal infections which are frequently epizootic in distribution. Many human streptococcal infections have their analogues in the lower animals, but fortunately these veterinary diseases are usually caused by species of streptococci with slight pathogenicity for man. Most group A streptococci likewise induce comparatively few animal diseases. Animal pathogens are usually members of group C

and, in the case of cattle, also of group B. Groups L and M (Fry, 1939) are probably pathogens of dogs and related species. Group D has pathogenicity for both man and animals, although several varieties often occur in healthy subjects. Its resemblance to *Str. lactis* and the avidity with which it invades the blood stream postmortem were formerly confusing factors in assessing its pathogenic role; yet with serologic and physiologic technics for its identification, it is clear that its ubiquity carries with it a definite threat to health. It is doubtful, however, whether group D infections often pass directly from an animal to a man, although such a possibility cannot be definitely eliminated.

Noteworthy is the difficulty of infecting domestic or laboratory animals through their respiratory tract with strains of group A, even though they be highly virulent; and when such respiratory infections are induced, the clinical symptoms are often mild or missing. Spontaneous wound infections of animals with group A streptococci occasionally occur; and pus from these wounds can be highly dangerous for man. In contrast may be cited the purulent lymphadenitis of guinea pigs due to "animal" group C strains. Enormously enlarged cervical lymph nodes break down and discharge pus which contaminates the food and bedding in the animal's cage with little danger to the caretakers, but with the probability that most guinea pigs in the same room will be infected. Similarly, strangles, a respiratory disease with purulent lymphadenitis of horses, induced by *Str. equi*, occurs without danger to the stable boys, veterinarians or to other species of animals, while spreading easily from horse to horse. The same is true of sleeping sickness and infectious peritonitis in chickens due to "animal" group C streptococci; whole flocks may be decimated without any similar illness occurring among the poultrymen.

Diseases induced in animals by parenteral inoculation with group A streptococci bear only a remote resemblance to human streptococcal infections. True, cutaneous abscesses result from subcutaneous inoculation, and when large doses or highly virulent strains are employed, there follow lymphadenitis and septicemia, but the clinical picture of human erysipelas is rarely reproduced. Intraperitoneal inoculation results in peritonitis and septicemia, just as it doubtless would in man. Indeed, the introduction of streptococci into animals through a hollow needle should be regarded as a wound infection which would be

expected to follow a clinical course comparable with similar human wound infections. The manifold clinical pictures that result from infections of man by group A streptococci are generally not seen in artificial inoculations of animals with the same bacteria.

One type of animal disease may have serious consequences for man: bovine mastitis due to group A streptococci. These strains are usually introduced into a cow's udder by a milker who is carrying virulent varieties in his nasopharynx; the udder becomes inflamed and excretes the human pathogens into the milk, which is mixed with that from other cows, and the whole lot, unless properly pasteurized, may infect large human populations. In fact this is the usual mode of establishing milk-borne epidemics of septic sore throat. At times, however, the milk is derived from quite healthy cattle but becomes contaminated by a streptococcus-carrying milk handler, with similar disastrous results. In contrast to the above picture, milk from cows having mastitis induced by groups B or C streptococci can be consumed by man with comparative safety. Fortunately for human health the latter forms of mastitis are much more common than the former, although from the standpoint of milk production they entail serious economic loss (Little and Plastring, 1946). Taken as a whole, the streptococcal diseases of domestic animals, fowls and bees doubtless cause marked financial loss to the agricultural industry. They are difficult to control because the microorganisms thrown off in nasal secretions, sputum, feces, milk and purulent exudates survive in the watering troughs, fodder, grain and bedding, and are easily transferred to other animals. Conditions are almost ideal for maintaining epizootics; and often the only way to stop them is to kill all infected stock, thoroughly disinfect the premises and reintroduce healthy animals. Streptococcal diseases of animals would seem to offer an ideal field for attempting active immunization, but relatively little work along this line has been done since the development of knowledge concerning serologic groups and types. Lee (1943) states that successful prophylactic vaccination has been attained in avian streptococcosis. Bazley (1940, 1942) has shown that with properly prepared vaccines from 4½-hour-old cultures of *Str. equi* horses can be made as solidly immune against strangles as are animals which have recovered from this disease. *Str. agalactiae*, on the other hand, does not induce in cattle such a solid immunity either by caus-

ing an attack of mastitis or when injected parenterally in the form of heat-killed vaccines.

## IMMUNITY

Early attempts to demonstrate common factors correlated with immunity to streptococci yielded many confusing results which are now partly accounted for by the existence of many different serologically distinct groups and types of streptococci. Moreover, the demonstration of several serologically separable components of streptococci opened the field to reinvestigation.

At least two definite forms of resistance have been demonstrated against immunologically different products of group A streptococci. First, antitoxic immunity directed against the erythrogenic toxin; and second, antibacterial immunity connected with the type-specific M component of these microorganisms.

Many features of scarlet fever are conditioned (a) by the capacity of streptococci to elaborate a peculiar soluble toxin and (b) by the ability of the patient to form an antitoxin against this poison. If, due to previous scarlet fever or to prior infections with streptococci producing relatively small amounts of toxin, a patient has acquired antitoxic immunity, he may be reinfected with a strong erythrogenic toxin-producing strain without developing scarlet fever because he possesses an antitoxin which neutralizes this toxin. If, on the other hand, the erythrogenic toxin elaborated by the reinfecting strain differs antigenically from that of prior infecting strains, then he may develop scarlet fever. Antierythrogenic toxin immunity can also be induced by actively immunizing "Dick-positive" individuals with bacteria-free erythrogenic toxin.

Immunity against erythrogenic toxin does not, however, insure freedom from infection with streptococci heterologous in type to those previously encountered. Antibacterial resistance is, moreover, closely associated with type-specific anti-M immunity. In ac-



tive or passive immunization of laboratory animals, the resistance to infection is closely connected, both qualitatively and quantitatively (a) with the M antigens produced by the streptococci in question and (b) with the antibodies directed against these substances.

Most patients infected with a given type-specific strain develop in their serum "bacteriostatic" antibodies, which are uniquely related to the type-specific M substance of the infecting strain and to no other M (Rothbard, 1945, extensive review and techniques). For example, a person recovering from type 19 streptococcal infection develops bacteriostatic antibodies against type 19 strains and not against those of heterologous types. Such bacteriostatic antibodies may be demonstrable in a patient's serum for many months and even one or two years; hence, such a patient will probably resist infection with streptococci homologous to that type for comparatively long periods. This hypothesis, however, has been tested in only a few instances, even though it has been substantiated in monkeys.

Such animals readily develop laboratory evidence of infection when inoculated intranasally with an M-containing virulent variant of group A streptococci, but not with a glossy, non-M-producing variant. For at least one or two years monkeys so infected resist reinfection with homologous type strains, while the same animals are easily infected with virulent heterologous type strains. Comparable evidence originates from the ease with which superinfections are induced in patients with scarlet fever or tonsillitis by strains heterologous to the primary infecting type. Such superinfections give rise to an appreciable number of complications.

While many adults have bacteriostatic antibodies against various group A streptococcal types in their sera, the average 3- to 5-year-old child has few or none, probably because he has experienced many fewer streptococcal infections than have adults.

This incidentally explains why children's leukocytes must be employed in bacteriostatic tests. Because early in the course of a group A streptococcal infection patients have no bacteriostatic antibodies in their sera against the homologous infecting strain, but may have these antibodies against one or more heterologous strains, it is logical to conclude that the antibodies they possess have resulted from prior infections with types heterologous to the current infecting strain.

The hypothetical quality of invasiveness which permits micro-organisms to pass the normal mucosal or cutaneous barriers and induce infections, is practically impossible to analyze in laboratory animals; its existence, however, can be inferred from infections in man. It may depend upon the dose of streptococci lodging in the mucosa, upon the elaboration of a hypothetical "invasive factor" or upon previous alterations of the tissue by other infections, viral or bacterial, or by physical or thermal traumata. Theoretically, the elaboration by many streptococcal types of a common "invasive factor" which might stimulate formation of a single antibody, would explain how infection with one type would enhance resistance to another.

One can also predicate that many streptococci elaborate in common pyrogenic substances, and also necrotizing enzymes, which kill tissues and induce formation of pus. Immunity induced by such an agent would then not be limited to any one serologic type, but the outcome of a given streptococcal infection might logically be partially conditioned by previous streptococcal infections. This might logically explain in part the changing age-linked picture in "streptococcosis."

Because antibacterial immunity to group A streptococci is closely connected with type specificity, it is understandable how one person might suffer many streptococcal infections each due to a different type, for there are about 40 recognized types and

probably many more as yet unrecognized. Probably, however, one individual rarely is infected with all the types to which he is exposed. The partial resistance to types heterologous to those which previously infected an individual seems to derive from factors connected with former infections. Indeed, in military streptococcal epidemics it has been repeatedly observed that recent recruits exposed to an invasive streptococcal strain become infected much more readily than do soldiers inducted several months or years previously.

The conception of "streptococcosis" advanced by Powers indicates progressively evolving modes of response to streptococcal infection from infancy through adolescence. The insusceptibility of infants under six months of age to the erythrogenic toxin differs from the resistance of most adults to this poison: regardless of the presence or absence of antitoxin in the mother's blood and without antitoxin in the baby's blood, the young infant's skin is evidently incapable of reacting to erythrogenic toxin, whereas an adult's cutaneous lack of responsiveness results from antibodies he has previously elaborated. The increasingly intense local response of the nasopharyngeal mucosa and the neighboring lymphatic tissues to streptococcal infection—slight in infants, more marked in children, and stormy in adolescents—can be attributed to a retuning of these tissues to similar stimuli. If one accepts the strict meaning of the word allergy as a "changed reactivity," then this retuning might be designated as allergy. But this word has commonly come to mean hypersensitivity or hyperergy; hence, the expression "retuning"—*Umstimmung*—more accurately expresses the changed reactive capacity. Such retuning is most strikingly illustrated in syphilis, and markedly so in tuberculosis; but in either disease one person is exposed to relatively few strains of treponemata or Koch's bacilli. With hemolytic streptococcal infection, however, there are exposures to many different serologic

types, each capable of inducing a new type-specific infection.

The usual absence of streptococcal type-specific antibodies from the blood of young children and their existence in that of adolescents and adults suggests that these antibodies may have arisen from repeated streptococcal infections. Part of the retuning may stem from tissue responses to streptococcal components other than the erythrogenic toxins or type-specific antigens. Indeed, the existence of such other antigenic, but non-type-specific, components has been well established; and development of antibodies against them might well account in part for the retuning under discussion. Theoretically, retuning might result in hypoergy to homologous-type streptococci and hyperergy to heterologous types. This subject has not been sufficiently investigated. The stormy, highly fatal streptococcal epidemics that occasionally sweep through isolated populations not previously exposed to streptococci suggest that the relative resistance now existing in most of the world has been induced by repeated exposure to many types of these micro-organisms.

Attempts to induce active immunity in man by injecting streptococcal vaccines or extracts have not been widely practiced since the development of knowledge concerning various antigenic components. Although such immunization seems theoretically possible, the existence of over 40 different types and the necessity of developing a corresponding number of type-specific antibodies, indicates the difficulties to be overcome in attempting to induce a comprehensive type-specific immunity. As already indicated, the existence of non-type-specific resistance is theoretically possible, but, as yet, it has been too little investigated to make logical attempts at vaccination feasible. For the moment, therefore, measures for controlling streptococcal infections must rely upon techniques other than those of active immunization.



## DIAGNOSIS

The protean manifestations of streptococcal infections indicate correspondingly diversified diagnostic measures. The special circumstances under which these infections thrive must be understood. The conditioning factor of age must be considered, not as an absolute entity, but as a pattern often seen from which many cases deviate. Such deviations are easily demonstrated in epidemics due to one strain of group A streptococcus; but their counterparts occur frequently in the general population where the contagious relationship of the various clinical pictures is difficult to establish.

Streptococci induce few clinical pictures which may not be evoked by other micro-organisms; hence, bacteriologic assistance is usually required for a definite diagnosis. Scarlet fever and erysipelas have enough clinical distinction to justify the conclusion that generally they are due to group A streptococci. Scarletina has been reported as having been caused by large colony varieties of group C streptococci and very rarely by staphylococci which produce an erythrogenic toxin.

A hemolytic streptococcal factor should be considered in all upper respiratory infections, although nonstreptococcal etiologies are relatively more common. Clinical features suggesting a group A streptococcal causation are: sudden onset with a chill or chilly feeling; distinct general malaise; fever of 102° F. or higher; leukocytosis of 12,000 or more with about 75 per cent polymorphonuclears; marked diffuse redness of the pharyngeal and posterior oral mucosa; yellowish exudate in small patches, most marked about the tonsillar crypts; marked enlargement of the tonsils, and of the lymph nodes below the angles of the jaw. The acute general symptoms which are shorter than the throat soreness, last four or five days and terminate abruptly. Nonstreptococcal pharyngitis, which has a more gradual onset with a lower fever and

very slight leukocytosis, often spreads downward to the trachea and bronchi; whereas, by contrast, in streptococcal sore throat the inflammation frequently spreads upward to the paranasal sinuses. An accompanying scarlatinal rash usually confirms a streptococcal etiology; and if the sore throat is followed within the next six weeks by hemorrhagic nephritis or rheumatic fever, a similar conclusion is justified. Persistent purulent nasal discharge indicates sinusitis, with a special obligation for the physician to search for streptococci because these exudates frequently spread the infections to others.

Postpartum fever always indicates a search for streptococci and for cultural and serologic identification of any strains recovered. If they belong to group A, a serious course may be anticipated; and if, from foul lochia, are grown anaerobic nonhemolytic varieties which form putrid gas, a grave outcome may ensue. Still other varieties, grown from the genital tract of a febrile woman following childbirth suggest infected retained membranes, the removal of which usually effects recovery.

In any purulent condition, efforts should be made to identify the causative micro-organism. Immediate microscopic examination of Gram-stained films of the exudate often suggests how the cultures should be treated. Gram-positive cocci in chains strongly suggest streptococci. Poorly stained cocci, with phagocytosis of some chains, indicate resistance on the part of the patient or relatively low virulence of the streptococci in question. If all of the cocci are extracellular, a relatively high virulence is suggested. If many micro-organisms are seen, a heavy growth is to be anticipated, and the exudate should be streaked very sparingly on blood agar so that the characteristic growth may develop in well separated colonies. If, on the other hand, few chains or pairs of cocci occur in the film, it is advisable to inoculate a tube of blood broth, incubate it for a few hours and from

this, inoculate blood agar where the characteristic colonies should develop. If the films show many streptococci but no aerobic growth occurs, anaerobic conditions should be provided. Indeed, some bacteriologists recommend routine anaerobic as well as aerobic cultures, and report a higher recovery rate of streptococci from exudates with this routine. If the microscope reveals two or more bacterial varieties in the films—in other words, a mixed growth—appropriate means must be taken to separate them. This may be possible by selecting well-separated colonies on solid media, but in making cultures of exudates from the nasopharynx, gastro-intestinal tract or skin it is sometimes advisable to use media containing small amounts of dyes or other materials which will suppress contaminants.

For primary isolation, a small amount of exudate or of broth culture of the exudate is spread with a suitable loop or other instrument on blood agar so that it is progressively diluted; thus, not only confluent but isolated colonies will grow. The exudate may be also inoculated into broth containing blood or serum in which the typical streptococcal morphology and chain formation most characteristically develop. After 24 hours incubation in blood broth, hemolysis of the red blood cells, browning of the sedimented erythrocytes with only a slight amount of overlying brown fluid, or unchanged sedimented blood cells, will suggest the appropriate categories of streptococci: hemolytic, green or indifferent.

Freshly poured blood agar plates should be employed to identify the characteristic colonial form of streptococci. Older plates are usable, provided they have been sealed with parafilm or other material during storage in the refrigerator, for it cannot be too strongly emphasized that the surface of the blood agar must be moist when inoculated, and also during the incubation period.

In making cultures of blood or seropurulent exudates, part of the material should be well mixed with good nutrient agar which

has been recently melted and cooled to about 42° C.; the mixture is then poured into warmed Petri dishes. Brown insists that for the development of characteristic beta, alpha, and alpha-prime hemolysis and of gamma colonies, the growth must have occurred in poured blood agar. The diagnosis of alpha and beta hemolysis is, however, usually made from growth on the surface of blood agar. Media for identifying the action of streptococci on blood should contain only a trace of sugar and should be well buffered because acid developed from sugar in the medium surrounding the colonies may confusingly alter hemoglobin.

Sheep blood agar is advised for culturing respiratory tract exudates, because on this particular medium the growth of hemolytic hemophilic Gram-negative bacteria is suppressed, while they grow readily on other blood agar. The clear zones about hemolytic streptococcal colonies on this medium have, however, a distinct greenish tinge which may be mistaken for alpha hemolysis. Concurrent growth on rabbit blood agar permits correct identification.

After 24 hours incubation at 37° C., the colonies on solid media should be examined with a hand lens in both transmitted and reflected light, and the colony appearance carefully noted. More detailed information is obtainable from a higher magnification. The surface characteristics of colonies are of value: first, as they may be compared with colonies of streptococci from other patients, because similar colony forms often occur among the streptococci from a single epidemic; second, in repeated isolations made over several months from the same patient, increasing visual glossiness suggests decreasing virulence of the strain which has infected this patient. The question of M antigen production by a group A streptococcus isolated on any one occasion can only be determined serologically, and it would be better in this respect not to allow visual appearance of colonies to influence the observer's opinion of the antigenic com



position of a strain. Again it should be mentioned that the phenomena of mattness and glossiness and of M and G functions (physiologic mattness and glossiness) has significance chiefly in describing variants of a single strain. If group A streptococci cannot be typed by the precipitin technic, their type may sometimes be determined by agglutination technics, particularly if they grow diffusely in broth when incubated either at 37° or 22° C. Avirulent, little M-producing varieties are seen more often in strains carried in the nasopharynges of patients long after recovery from a streptococcal infection (Rothbard, 1948); similar variants also occur in strains maintained for a long period on artificial media.

Matt or mucoid colonies are often ovoid in outline from a flowing of the mucoid material over the agar. Sometimes transition of a watery into a matt surface may be observed; again the watery appearance persists, and its mucoid qualities are demonstrable by a stringiness on a loop slowly withdrawn after touching a colony, and by large capsules seen in India-ink preparations. Other surface topographic features, such as stippling, raised margins, central nipples or craters, and much or little pericolonial hemolysis, are all characteristics which help in identifying particular strains or types. A constancy of these features in cultures from several patients helps at times in determining the epidemic-inducing character of certain strains.

The action on blood of a strain in question is the primary identifying feature, although there are both hemolytic and non-hemolytic members of several groups, particularly of B and D. Because many group B colonies form yellowish pigment, one should be on the lookout for its presence. As the brownish or yellowish color of blood agar may mask its presence, when it is suspected, a subculture may be made in starch broth in which it is more evident, and the color of the sediment of centrifuged broth cultures should also be noted.

Streptococci under examination should be classified according to their serologic, physiologic, and biochemical characteristics. Modern bacteriology requires at least identification of the serologic group to which a strain belongs, and the serologic type must be determined for epidemiologic studies. Streptococci for preparing extracts must be grown in broth in which the C and M substances are known to be elaborated, for heavy growth may occur without M substance being detectable. The streptococcal proteinase which destroys M protein is usually inhibited by using neopeptone, instead of other peptones, in the medium.

If the streptococcus belongs to a group other than A, its physiologic traits may permit of further division; this is particularly possible with members of groups C, D and N. (See Table 29.) Moreover, certain streptococci have definite enough physiologic characteristics to permit placing them within a group without resorting to serologic technics, but much time and expense can be avoided if primary group classification is determined serologically. No system of classification and identification so far devised covers the whole multitude of streptococci. A combined study of their serologic and physiologic peculiarities is the best that current knowledge of these micro-organisms can offer, and such a combination is still requisite for satisfactory bacteriologic diagnosis.

## TREATMENT

Because streptococcal infections occur under numerous circumstances and affect many different organs, no single therapeutic plan is universally applicable. Modern antibiotics now comprise our most useful curative weapons. Among them, penicillin is the most effective; but all streptococci are not equally susceptible to its influence. The enterococci and the viridans strains designated SBE are especially resistant, although cures may be effected with doses much higher than those usually employed.

The sulfonamides often quickly induce recovery and possess the advantage of being effective when taken orally. Sufficient drug must be given to maintain a blood concentration of 8 to 10 mg. per cent. The least toxic forms, such as sulfadiazine, are recommended. Therapeutic failure with these drugs sometimes is due to sulfonamide resistance of the strains in question. Such strains occurred in epidemics in military installations where sulfa prophylaxis had been applied extensively. The few sulfa-resistant strains encountered in civilian practice probably derive from military infections or carriers.

Because drug-resistant strains may rapidly evolve in streptomycin-treated patients, this drug should be reserved for special cases where a failure is encountered with the two usually efficient antibiotics, sulfonamides and penicillin.

Tyrothricin is quite useful against superficial streptococcal infections if it can be applied directly to the diseased area without being absorbed into the blood stream. In suitable vehicles it is especially applicable to superficial streptococcal infections of the skin and upper respiratory tract.

With any antibiotic it is necessary to bring the drug into contact with the streptococci in sufficient concentration to effect bacteriostasis. In meningitis and purulent infections of the serous cavities and joints, direct injections of the remedies into the infected cavity after evacuation of the pus is indicated. With infected wounds, when accompanied by crushing or laceration of the tissues, surgical débridement is requisite. Purulent osteomyelitis often requires surgical drainage and excision of the necrotic bone.

In treating acute streptococcal endocarditis, penicillin is the drug of choice, and must be given intravenously in large doses until the bacteremia is permanently obliterated. In subacute streptococcal endocarditis the dense exudate presents a barrier to the circulating antibacterial substances and

the offending micro-organisms are often specially resistant to the chemotherapeutic agents; hence, therapy should be both intensive and prolonged. In vitro testing of the resistance of the streptococcal strain in question to the drug one anticipates employing often helps in deciding on the correct form of therapy. Sulfonamides have not proven very satisfactory in this disease, although they sometimes sterilize the blood for appreciable periods, but bacteremia usually recurs when they are discontinued. Penicillin, on the other hand, is much more effective if large doses are given day and night, and the treatment continued many weeks so that the streptococci deep seated in the endocardial vegetations are constantly exposed to high concentrations of the drug. Obviously, however, those patients who recover from active bacterial endocarditis will still have valvular deformities, usually more marked than prior to the onset of the disease.

While antibiotic drugs are usually very beneficial in both primary streptococcal infections and in complications induced by the original strain or by strains belonging to other types, they are comparatively ineffective in the treatment of such sequelae as nephritis and rheumatic fever. It is important to understand the differences in these time factors when these drugs are administered. During the phase of the sequelae, the toxic effects of sulfonamides when superimposed on those of rheumatic fever or nephritis may aggravate the symptoms. Such combined deleterious insults are less frequently encountered in penicillin-treated patients. Occasionally, following penicillin therapy, the allergic after effects of this drug, particularly arthralgia, may confuse the picture and suggest the presence of poststreptococcal rheumatic fever, when in fact the symptoms have been induced by the drug. There is, of course, no valid reason why drug allergy and poststreptococcal sequelae may not exist simultaneously.



Some physicians claim that neither sulfonamides nor penicillin given in streptococcal nasopharyngitis, particularly in tonsillitis and scarlet fever, materially shorten the course of these diseases; nevertheless, considerable symptomatic relief is often effected by these drugs as shown by a precipitous drop in the fever and rapid amelioration of the toxic symptoms. If penicillin is given very early in the course of scarlet fever, the rash, fever and toxic state may rapidly disappear, and the antitoxic immunity which ordinarily develops at the end of the first week may fail to develop. In such patients, reinfection with a markedly rash-inducing strain may cause a second attack of scarlet fever.

A patient treated with antibiotic drugs may show a satisfactory and rapid recovery from the primary streptococcal infection but still develop such sequelae as rheumatic fever or nephritis. There are indications that very intensive antibiotic therapy started early, i.e., during the first to third day of an acute streptococcal infection, will prevent these serious sequelae. If the sequelae result from the development of peculiar antibodies which react with antigenic residua from the streptococci, then the prevention of the development of such antibodies by early and intensive antibiotic therapy might conceivably prevent these sequelae. This hypothesis is now being tested.

#### EPIDEMIOLOGY AND PROPHYLAXIS

Prophylaxis of streptococcal infections in man necessitates fulfillment of these conditions: (1) preventing pathogenic strains from reaching vulnerable areas; (2) increasing the resistance of such susceptible areas to infections by these pathogens. The first desideratum is very difficult to attain because streptococci are widely disseminated; some degree of success is possible with respect to the second.

In group A streptococcal infections the ultimate source of the pathogenic agent is usually a person who is eliminating them from diseased foci. Such foci may be clinically obvious, inaccessible to inspection, or apparently normal, as in the case of so-called "healthy carriers." There is increasing evidence that such "healthy carriers" often have subclinical upper respiratory infections. Persons suffering from acute streptococcal infections obviously eliminate virulent streptococci from the diseased areas, either directly into the air, or upon their bodies, especially their hands; also on clothing, bedding, lint and dust derived from such fomites. In upper respiratory infections, minute droplets of mucus, forcibly expelled from the mouth and nose by coughing or sneezing, may serve as vehicles which transfer the pathogen directly to another person, but the transfer is more frequently effected through dust, lint or other fomites on which streptococci may survive for weeks in a dried state. A handkerchief containing nasal exudates usually contaminates the hands, clothing and especially the pocket, and lint therefrom reaching the floor deposits streptococci there. The blankets and sheets used by such a person are rich depositories of streptococci, as are surgical dressings covering a streptococcal infected wound. Any of this contaminated cloth, when agitated, gives rise to polluted air, especially when shaken in bed making, changing dressings or in other maneuvers. Dust raised from sweeping rooms or wards occupied by these patients is a potent source of infection by streptococci carried in the clouds of dust. Oiling of blankets to decrease the throwing off of lint and of floors to retard raising of dust are quite effective measures in decreasing pollution of air. Proper ventilation with filtered, warm, moist air admitted at the top of operating or dressing rooms and expelled from the bottom is useful in keeping the streptococcal content of air at a low concentration.

Sufficient time between dressings and operations is required to permit effective clearing of air. Ultraviolet light and aerosol mists are likewise effective when properly regulated, but all such measures require expensive equipment not readily available.

Nasal discharges are probably more dangerous than saliva, primarily because the disposal of sputum is usually more effective, and secondarily because even with slight clinical manifestations, hemolytic streptococci often exist in heavy concentrations in exudates from infected noses and sinuses; and the carrier state may not be detected in throat cultures (Hamburger et al., 1945). In fact, determining the number of streptococci which a patient throws off is probably more important than merely learning whether or not he is a carrier, because it has been definitely demonstrated that a patient may yield a few streptococci in nose or throat cultures with relatively little danger to his human contacts. The time since the patient was infected is also important with respect to the nasopharyngeal carrier state, as has been well established by the small number of "returned cases" seen in contacts of patients who have been released from three weeks' quarantine after having had scarlet fever. Indeed, the number of streptococci disseminated by a convalescent and the time since his infection are probably more important features to be considered in allowing him to circulate freely, than is the establishment of any fixed rule in which these factors are disregarded.

Because a disseminator of streptococci is dangerous to his environment in proportion to the concentration of these micro-organisms he is discharging, effective measures which decrease this concentration are correspondingly prophylactic. Modern antibiotics have proven useful for this purpose, provided the streptococci in question are susceptible to the drugs employed. Sulfadiazine, 1 gram per day, will markedly re-

duce or completely eliminate group A streptococci from the sputum and nasal discharges of many patients after three or five days, but the cocci often return, though in much smaller numbers, after the drug is discontinued. Penicillin, in sufficient doses, more quickly suppresses the streptococci, and when given intramuscularly for from 10 days to two weeks in vehicles which prolong its action, permanently eliminates the streptococci from most patients' noses.

Increasing a patient's resistance to group A streptococci by prophylactic technics based on immunologic procedures has not been effectively developed. Sulfadiazine, on the other hand, increases the resistance of many persons sufficiently to protect them from ordinary infection with group A streptococci. Most people can take this drug for long periods, but all such patients should be carefully observed for toxic drug action. Under special epidemic conditions, the widespread utilization of sulfonamides both decreases the output of streptococci by the carriers and increases the resistance of the susceptibles; but one should always be conscious of the danger of allowing drug-resistant variants to appear when such prophylactic technics are employed. Penicillin has a similar prophylactic influence, but the necessity for parenteral injection, or for using large doses by mouth, makes its prophylactic application less feasible than that of the sulfonamides. Newer forms of penicillin may resolve these objections. The simultaneous employment of two different antibiotics increases the effectiveness of such forms of prophylaxis.

Except for those diseases induced by anaerobic varieties, most streptococcal infections in a given individual originate from strains heterologous to those carried by him, and a person may carry virulent strains without harm until they are introduced into his tissues by some trauma or by another infection which lowers his resistance. Thus, an attendant of a patient with scarlet fever



or puerperal sepsis may effectively resist pathogenic strains on his hands until a needle prick or a scratch carries them through the skin. Then infection occurs. Likewise, streptococci carried harmlessly in a parturient woman's nose may, on transfer to her genital tract, give rise to a serious postpartum sepsis. The potential threat to a parturient woman from vaginal carriage of anaerobic streptococci has been stressed.

The various special circumstances under which streptococci induce infection should be kept in mind, and prophylactic measures suitable to the particular circumstances instituted. Tissues vary in their vulnerability. A superficial wound may be safely exposed to an atmosphere containing a few streptococci which would be dangerous for opening the pleura or meninges. A granulating wound resists infection more effectively than a fresh one. Large areas resulting from burns are more easily infected than small wounds. In the puerperal state and in conditions where unusual susceptibility to streptococcal infections exist, special precautions should be taken to avoid contact with streptococcal carriers or to an atmosphere containing potential pathogens.

Persons having rheumatic cardiac disease should be protected against streptococcal infections, because group A infections often induce rheumatic recurrences which increase the cardiac damage. Because streptococci circulating in the blood are prone to lodge on deformed valves (usually rheumatic in origin), and thus induce bacterial endocarditis, and also because nonhemolytic streptococci frequently enter the blood temporarily following operation on the mouth or upper respiratory tract, it is wise to place these cardiac patients under the influence of an antibiotic drug—preferably penicillin—just prior to tonsillectomy or tooth extraction. This will usually insure that these micro-organisms which enter the blood will be killed before they might infect the deformed valves.

SPECIAL MEDIA AND SEROLOGIC TECHNICS

TODD-HEWITT (1932) BROTH (MODIFIED)

**Beef Meat Infusion Base.** Cut away as much fat as possible from fresh beef heart or horse meat. Chop or grind the lean meat fine and to each pound add 1,050 cc. of distilled water. Stir, and with a sieve skim off the small particles of fat which rise to the surface. Place the meat and water mixture in the ice box overnight. The next morning heat to 85° C. and maintain this temperature for one-half hour. Filter the broth through coarse filter paper.

To each liter of the above infusion add 20.0 Gm. of neopeptone. Adjust pH to 7.0 with N/1 NaOH and add the following:

NaCl.....	2.0 Gm.
NaHCO <sub>3</sub> .....	2.0 Gm.
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O.....	1.0 Gm.
Glucose.....	2.0 Gm.

Boil for 15 minutes and filter through filter paper. Tube (in 40 cc. amounts \*) and sterilize in the Arnold for one hour on 3 successive days. The final pH should be 8.0. The 15 minutes boiling should drive off the CO<sub>2</sub> prior to Arnolding. If not, a precipitate may be formed during sterilization. If this happens, the broth must be filtered and the pH re-adjusted under sterile conditions, followed by one hour in the Arnold. The anti-M proteinase is inactive in this broth due to the use of neopeptone.

Blood broth for stock cultures may be prepared by adding two or three drops of defibrinated rabbit or sheep blood to 5 cc. of the Todd-Hewitt broth.

DIALYSATE MEDIUM (DOLE, 1946)

Dole (1946) prepared a medium composed of dialyzable components which contains a minimum of antigenic materials usually present in broth made with unpurified peptone and meat extract; it supports a rapid growth of group A streptococci. It is prepared as follows:

**Beef Heart Dialysate.** Beef heart—strip off fat, grind fine and weigh; to each pound (454 Gm.), add 250 cc. of water and soak overnight at 4° C.; heat mixture carefully at

\* For facilitating later manipulation, it is convenient to tube the broth in 50 cc. centrifuge tubes

85° C. for 30 minutes; filter through fluted filter paper (supported under point with cone of cheese cloth), and express juice with wooden masher; cool by immersing receiving flask in running water. Place filtrate (about 300 cc.) in a 1-meter length of cellulose sausage casing; \* place casing in upper two-thirds of a tall narrow vessel; place 600 cc. of water in vessel outside of casing; dialyze 12 to 18 hours at 4° C.; remove and save dialysate in refrigerator; replace water and dialyze 12-18 hours at 4° C.; repeat dialysis a third time; combine dialysates. These may be used immediately or concentrated *in vacuo* to about one-twentieth the original volume. Place in refrigerator overnight; then separate from crystalline deposit by centrifuging and pipetting off the supernatant fluid. The concentrate may be preserved by freezing in plastic containers in dry ice box or by freezing and drying in a high vacuum. In the latter form, about 8 Gm. is obtained per pound of original meat. Use either 1 Gm. of this powder or one-eighth of frozen concentrate for each liter of medium.

**Peptone.** Pfanstiehl, Difco proteose or neopeptone yield the largest growth. Purification to remove color: dissolve in water at 80° C. to make 20 per cent solution; add activated charcoal 50 Gm. per liter of solution; hold at 80° C. for 1 hour with stirring; filter hot through soft filter paper with aid of suction; dialyze 3 times the same as the beef heart extract; adjust pooled dialysate to pH 8.0; adsorb with 50 Gm. charcoal per liter of dialysate overnight at 4° C.; filter; concentrate filtrate *in vacuo*; then reduce to a powder by freezing and drying.

*Preparation of complete medium (solid content 2.3%):*

- (A) Peptone: 15 Gm. (or dialysate from 20 Gm. of original peptone)

NaCl: 2 Gm.

Na<sub>2</sub>HPO<sub>4</sub> (anhydrous): 0.5 Gm.  
(Omit if neopeptone is used.)

Water: 900 cc. Adjust reaction to pH 8.0 with N/1 NaOH; make up to 950 cc. with water.

Sterilize by heat or filtration.

- (B) Beef heart extract 1 Gm. powder (or concentrated dialysate from 1/8 pound of meat).

Glucose: 2 Gm.

Water: make volume 50 cc.; adjust reaction to pH 8.0.

Add NaHCO<sub>3</sub>: 2 Gm.

Sterilize by filtration through a por-

celain filter. Combine solutions A and B and inoculate immediately. If delay in inoculating medium is anticipated, omit adding the NaHCO<sub>3</sub> until just before using; then add a sterile filtered solution containing 2 Gm. of sodium bicarbonate.

As this medium filters almost as readily as water, weighed amounts of the components may be dissolved in proper amounts of water, then adjusted to pH 8.0, the NaHCO<sub>3</sub> added, and the entire mixture sterilized by passing through a porcelain filter.

#### TECHNIQUES OF GROUPING AND TYPING STREPTOCOCCI

**Hydrochloric Acid Extracts of Streptococci for Precipitin Grouping and Typing Tests.** Inoculate 40 cc. of Todd-Hewitt broth (if possible in a 50 cc. centrifuge tube); incubate for 18 to 24 hours; check purity of growth; centrifuge, and discard supernatant broth.

To sediment add 0.4 cc. N/5 HCl made in normal saline solution. A loopful of suspension should give an orange-red color with a drop of 0.01 per cent thymol blue. A pH of 2.0 to 2.4 is needed. Place mixture in pointed 15 cc. centrifuge tube; heat in boiling water bath for 10 minutes, shaking every three minutes; cool and centrifuge. Decant supernatant fluid into a second centrifuge tube; add 1 small drop of 0.01 per cent solution of phenol red, which colors the fluid yellow. Add drop by drop 0.3 to 0.33 cc. of N/5 NaOH until faint pink color appears. When near end point of pH 7.0, adjustment of reaction is facilitated by employing N/20 NaOH and N/20 HCl. Final reaction should be between pH 7.0 and 7.8. (The HCl and NaOH solutions should be kept in pyrex containers.)

Centrifuge so that extract is water clear. If used immediately, it may be kept in centrifuge tube, but if not, it should be decanted into a small tube. Cloudy extracts should not be used.

This extract may contain both group-specific C and type-specific M antigens. If one is interested only in grouping the streptococci in question, or if in doubt concerning the grouping results with the hydrochloric acid extracts, a formamide extract may be prepared (Fuller, 1938).

**Formamide Extract.** Centrifuge from 10 to 15 cc. broth culture until the bacteria are

\* Visking Cellulose Sausage Casing, "NoJax" 27/32.



packed; remove the supernatant as completely as possible and discard; to the sediment add 0.2 cc. of formamide. Shake; and place the tube in an oil bath (automobile or mineral oil) at  $150^{\circ}$  to  $180^{\circ}$  C. for 15 minutes; cool; and add 0.5 cc. of acid alcohol (1 cc. concentrated HCl in 99 cc. of 95 per cent alcohol); and centrifuge. Transfer the *supernatant* to a

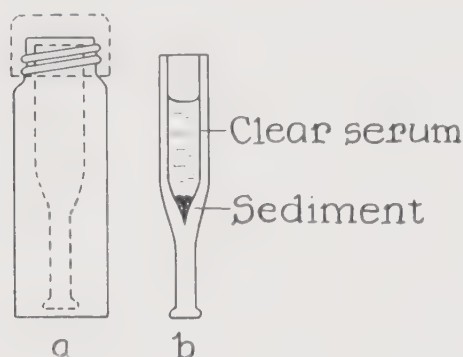


FIG. 7. Serum container. (a) Screw cap vial showing position of serum cup; (b) details of serum cup containing serum with underlying sediment.

clean centrifuge tube; add 1 cc. of acetone and centrifuge lightly; discard the supernatant. Add 2 cc. of normal saline to the *sediment*; shake, and add 1 drop of bromthymol blue indicator; then add sufficient 2 per cent sodium carbonate ( $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ , not "technical" sodium carbonate) to turn the extract blue. Centrifuge before use in the precipitin test.

**Apparatus.** Capillary pipettes for *typing* are made from stock capillary tubing \*  $1.0 \pm 0.2$  mm. external diameter, broken into 7 cm. lengths. The external surface is cleaned with soft tissue paper; the inner surface is not cleaned. Pipettes are placed in test tubes which are capped with *unsized* paper and sterilized by dry heat.

Capillary pipettes for *grouping* are made from tubing  $1.5 \pm 0.2$  mm. external diameter. The middle of a 12 cm. length is melted in a narrow flame and drawn quickly so that fine points are formed. Thus, two conically pointed

pipettes are made with fine openings about the diameter of a hair (Fig. 10). The tubes are cleaned and sterilized as described above.

Serum containers consisting of an inner goblet-shaped cup and an outer screw capped vial, as shown in Figure 7, are sterilized in an autoclave; then the cups are filled from stock serum bottles. Sediment collects in the bottom of the cup as shown in Figure 7 and must be neither disturbed nor drawn into the pipettes in performing the test. These serum containers are mounted with plasticine in suitable blocks (Fig. 8).

**CAPILLARY PIPETTE STAND** (Fig. 9). This consists of a wooden block 10 inches long,  $1\frac{1}{4}$  inches wide and  $\frac{7}{8}$  of an inch deep. A groove  $\frac{1}{4}$  inch wide and deep is cut lengthwise on one side, and is filled with plasticine. Labels are written on narrow strips of ruled paper and fastened in front of the groove. One stand is used for each extract when the complete set of sera is employed.

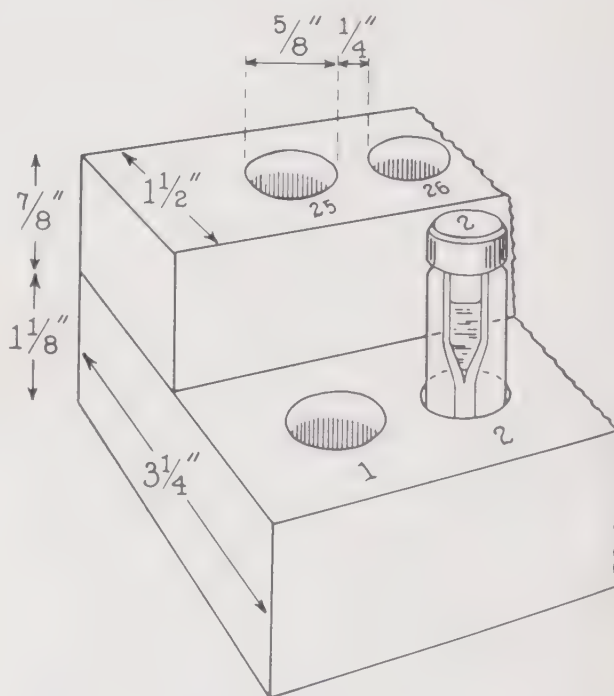


FIG. 8. Wooden block for holding serum containers. The length of the block can be arranged to fit available refrigerator.

For grouping tests the plasticine is formed in a roll above the surface of the block, as shown in Figure 10.

**Reading Equipment.** A dull black screen is placed beneath and a few inches behind an electric light so that the tubes may be observed with back-lighting against a black

\* The capillary tubing is made by the Kimble Glass Company, Vineland, New Jersey, and can be obtained from laboratory supply houses. It is described by that Company as No. 46485 capillary pipette tubing, made of neutraglas (N-51A glass), not individually gauged, and varies in outside diameter from 0.7 to 1.0 mm. with 0.2 mm. wall. There are approximately 500 thirty-four inch lengths to the pound. For confirmatory tests and group classification, larger tubes with outside diameter of 1.2 to 1.5 mm. are used; 1 pound contains approximately 300 thirty-four inch lengths.

background. A fluorescent lamp is superior to an ordinary incandescent bulb. The tubes are examined with a hand lens of about 5 diameters magnification.

**CAPILLARY PIPETTE GROUPING** (Swift, 1947). Grouping may be performed either for final identification, or as a preliminary step in identifying members of the various groups.

The bacterial extract, serum vials, capillary pipette stands, 1.5 mm. conical ended pipettes are arranged conveniently. The sterile pointed end of the pipette is dipped into the grouping serum until a column about 1 cm. long has been slowly drawn in by capillary action. This end of the pipette is wiped with paper tissue, and then dipped into a drop of extract until an equal amount has been drawn into the pipette. Air bubbles must not separate serum and extract. The pipette is wiped, the conical end sealed by plunging it into a lump of plasticine. The lower open end is pressed against

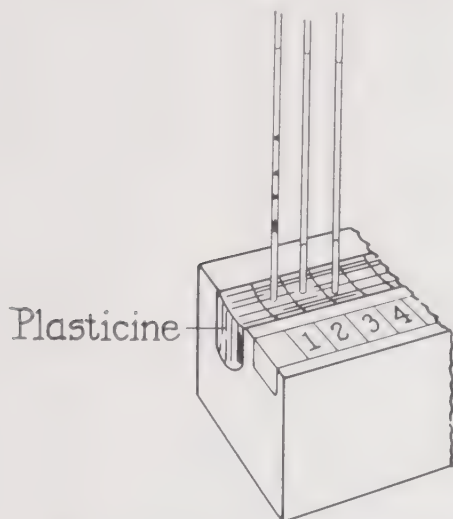


FIG. 9. Capillary pipette stand arranged for typing. Precipitate in capillary pipette 1 indicates a positive reaction with type 1 serum.

the roll of plasticine and thus the pipette is held in a vertical position without being plunged into plasticine; in this way fluid is not forced out of the pipette by hydraulic pressure, and the column of fluid does not move. The end containing the fluid must not be touched by the fingers. This technic insures the least possible mixing of the underlying serum and the overlying extract, and at the interface between these two reagents the formation of precipitate is observed. If much movement occurs, there is rapid mixing of the reagents, and the reactions may be so inde-

terminate as to require confirmation in small test tubes. If very potent grouping sera are available, simple 1.5 mm. capillary pipettes may be employed. In this case the pipette is so mounted that the serum is below and the extract above. A minimal movement of the column of fluid is desirable.

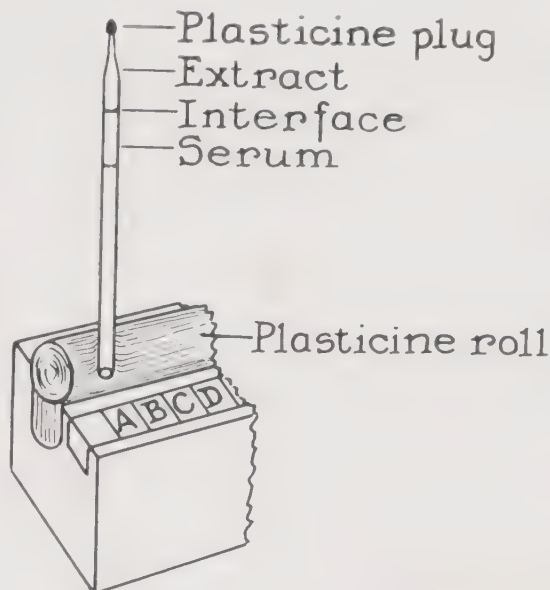


FIG. 10. Capillary pipette stand for conically ended grouping pipettes. Pipette is held vertically by pressing against plasticine roll, lower end of pipette open.

*Reading.* Within 5 or 10 minutes a positive reaction is shown by a cloudy white ring of fine precipitate at the interface. With weak reactions longer time may be required. After the pipettes are incubated at 37° C. for an hour, weak cross reactions with other sera may occur; hence readings made within 10 minutes usually indicate more specifically the group to which the streptococci under examination belong.

If confirmation is required, place ordinary medicine droppers, with the bulbs removed, vertically in a plasticine block so that the small ends are sealed. Place 0.05 cc. of proper serum in each and slowly pipette an equal amount of extract into each tube so that it layers over the serum. Make readings within 30 minutes: a white ring at the interface indicates a positive reaction. If reactions are not clear cut, make extract dilutions of 1:4, 1:8 and 1:16 and test in a similar way. If cross reactions still occur, make formamide extract and retest.



**CAPILLARY PIPETTE TYPING.** Only extracts are tested which have reacted positively with group A serum. The sterile end of a 1.0 mm. capillary pipette is placed in the serum until a column about 1.5 cm. long has been drawn in by capillary action. Wipe the outside of the wet end and dip it into the extract and allow an equal column to run in after the serum, with precautions not to allow a bubble of air to separate the two reagents. The column is allowed to run to the middle of the pipette which is carefully wiped with soft paper tissue. It is then inserted into the plasticine so that the serum is above the extract. Similar preparations are made with each serum.

**Reading.** The tubes are immediately examined for cloudiness with a hand lens and if foreign particles are present or the pipettes are dirty, they are discarded and the test repeated. After 2 hours' incubation at 37° C., preliminary readings are made. A final reading is recorded after the pipettes have stood overnight in the refrigerator. Most positive reactions appear after two hours' incubation. If, in the interest of economy, a number of extracts have been set up with only one type serum and negative results have been obtained, the extracts may be set up immediately with all the other type sera. The following scale is used:  $\pm$ , just visible; +, a few fine masses visible with the hand lens; ++, usually beaded throughout, visible with the naked eye; +++, and +++++, column filled with larger masses of precipitate. Each positive test must be interpreted in the light of the known reaction of the serum with homologous extract. One plus or weaker readings should not be accepted as diagnostic.

With properly absorbed sera, true cross reactions are rare. Various doubtful positive reactions result from poorly prepared extracts, cloudy, contaminated sera or extracts and from dirty tubes. When a particular test is doubtful, it should be repeated again with serum, saline and serum, and homologous extract controls. Confirmatory tests may be performed with larger pipettes or small test tubes and with several different dilutions of extract.

In carrying out the precipitin tests, the action of the sera must be known because they are not uniform in strength or purity: some give quick reactions, some slow; and some

give slight cross reactions. Acquaintance with these individual peculiarities will prevent confusing interpretations. Although over 40 types have been identified among group A strains, still many untypeable strains are encountered among civilian streptococcal infections. During World War II, over 90 per cent of the strains recovered were included in 12 to 15 types.

**Slide Agglutination Technic.** **BACTERIAL SUSPENSIONS.** The chief technical difficulty in agglutination tests with streptococci is the tendency of many strains to undergo spontaneous agglutination. Much study has been given to overcoming this tendency, but no universally applicable method has been found. The use of 1 per cent serum in the broth sometimes helps, but occasionally streptococci grown in serum broth become inagglutinable. Adding varying amounts of glucose to the medium may yield satisfactory suspensions. Growing the cultures at room temperatures sometimes yields stable suspensions. Often none of these maneuvers is required.

Suitable broth cultures are centrifuged; and the sediment is suspended in normal saline to make heavy suspensions of a standard density, approximately twenty billion microorganisms per cc. (For a preservative, add 0.5 per cent phenol.)

**SLIDE AGGLUTINATION TESTS.** These are made at room temperature on ordinary microscopic slides upon which 10 or 12 squares (each about 1 cm. in diameter) have been ruled with a grease pencil. With a loop about 2.5 mm. in diameter, a loopful of properly absorbed serum is placed on a square; then a similar loopful of bacterial suspension. The two drops are mixed, the slide rocked and rotated every few seconds, and the results read against a dark background under a suitable light. They are also read microscopically with a low-power objective. The final reading is made at the end of one minute. A suitably prepared control with normal serum is always set up. The differences between the agglutination with normal serum or with heterologous serum and the homologous type-specific serum should be observed within 10 to 20 seconds. The reactions should also be checked with a streptococcal suspension of known type and its homologous serum.

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# 12

## The Mycobacteria

### INTRODUCTION

The mycobacteria constitute a genus (*Mycobacterium*) of the family, *Mycobacteriaceae*, of the order, *Actinomycetales*. The mycobacteria are usually spoken of as acid-fast bacilli; they do not stain readily, and, once stained, they resist decolorization by strong acids or by alcohol. The pathogenic mycobacteria include the tubercle bacilli (*Mycobacterium tuberculosis*), the Johne's bacillus (*Mycobacterium paratuberculosis*) and the leprosy bacillus (*Mycobacterium leprae*). There are many species of nonpathogenic mycobacteria: for example, the smegma bacillus (*Mycobacterium smegmatis*) found on man, the timothy bacillus (*Mycobacterium phlei*), the butter bacillus (*Mycobacterium butyricum*) and others. The property of acid-fastness is also possessed by some aerobic types of *Actinomyces bovis*.

The pathogenic mycobacteria cause chronic diseases with lesions of the infectious granuloma type. The fully developed lesions of tuberculosis, leprosy and Johne's disease (a chronic enteritis of cattle) have in common certain histologic characteristics: collections of epithelioid and giant cells are conspicuous, but the type of necrosis called caseation occurs only in tuberculosis.

### MYCOBACTERIUM TUBERCULOSIS

#### HISTORY

The tubercle bacillus causes such a wide variety of lesions and clinical symptoms in

man and animals that, before its discovery, the pathologist and clinician long remained unaware of the common etiology of diseases such as miliary tuberculosis, tuberculous caseous pneumonia, tuberculosis of cervical lymph nodes (scrofula), and lupus vulgaris. The modern knowledge of tuberculosis may be said to have begun with Laennec, who, in 1819, described some of the macroscopic aspects of tuberculous lesions stating that the lesions can occur both as isolated "follicular" (miliary tubercles, caseous tubercles) and as "infiltrative" forms (exudative tuberculosis). He recognized the essential unity of early, semitransparent tubercle and of the caseous tuberculous lesions. Villemin, in 1865, demonstrated that material from the human tuberculous lung produces tuberculosis in the rabbit, and later he transmitted tuberculosis from cattle to rabbit.

The discovery of the tubercle bacillus by Robert Koch in 1882 was preceded by the discovery of the leprosy bacillus by G. A. Hansen in 1878. These discoveries were made before the acid-fast tinctorial properties of these microbes were known. The mycobacteria were stained by immersing the preparations for 24 hours in aqueous alkaline methylene blue solutions. Ehrlich, in 1882, showed that in the presence of aniline



oil the tubercle bacilli can be stained with basic dyes and remain stained after treatment with strong nitric acid. The method currently used to stain acid-fast bacilli was developed by Ziehl, in 1882, and subsequently slightly modified by Neelsen.

#### MORPHOLOGY

Tubercle bacilli occur as rod-shaped micro-organisms in animal lesions. On artificial cultivation they vary from coccoid to long filamentous forms depending upon the type (bovine, human, avian, etc.), the particular strain, the age of the culture and environmental conditions. The typical rods are straight or somewhat bent with parallel sides and rounded ends; in the animal body they vary in length from 1 to 4  $\mu$  and from 0.3 to 0.6  $\mu$  in diameter. No capsule has been demonstrated on the tubercle bacillus either in susceptible hosts or on artificial media.

The most characteristic staining property of the true tubercle bacilli is their acid fastness. Ethanol containing 3 per cent hydrochloric acid (acid alcohol) will decolorize all rod forms of other bacteria within a few minutes, but the tubercle bacilli will resist this treatment for many hours. Young forms are somewhat less acid fast than older forms. The acid-fastness of the tubercle bacillus is dependent upon the integrity of cellular structure since physical trauma is sufficient to render it non-acid-fast and readily stainable by an aniline dye (Yegian and Porter, 1944).

The nonpathogenic mycobacteria are less acid fast than tubercle bacilli, but are difficult to decolorize with alcohol alone; they are, therefore, more properly designated alcohol fast.

Mycobacteria cannot be classified as Gram positive or Gram negative by the Gram staining technic because once they have been stained by basic dyes, they cannot be decolorized by alcohol regardless of whether or not they have been treated with iodine (Kretschmer, 1934).

It has been claimed that tubercle bacilli have a life cycle including a very small viable, granular form which can pass through ordinary bacteriologic filters. These claims are unsubstantiated (Pinner et al., 1931).

#### CULTIVATION

Human, bovine, avian and murine strains of tubercle bacilli can be grown under aerobic conditions on a variety of simple synthetic or complex organic media, between pH 6.0 and 8.0 with an optimum around 6.5 to 6.8; they can utilize a multiplicity of carbon compounds as source of energy, in particular, glucose and glycerine, and of amino acids as source of nitrogen. Growth is enhanced by the dicarboxylic acids and by the amide asparagin, but there is as yet no evidence that any of the known vitamins behave as growth factors for these organisms. Growth is most rapid at from 37° to 41° C. but, even then, is very slow in comparison with that of common bacterial species; the fastest growth rate recorded in the literature corresponds to a division time of 36 hours in the case of the human strain H37Rv. This time has been cut approximately in half in media containing serum albumin and fatty acids or synthetic fatty acid esters (Dubos et al., 1947). Growth of avian strains is much more rapid, particularly in the latter type media.

Two very different types of media have been in general use for the cultivation of tubercle bacilli. Simple synthetic media containing an abundant supply of minerals (in particular, phosphate, magnesium and iron ions), of dicarboxylic acid (or of arginine) and of glucose or glycerine have proven capable of supporting the synthesis of large amounts of bacterial protoplasm. Thus, the medium described by Long and Seibert (1926), which has been widely used in the United States, can give bacterial yields upward of 10 Gm. (dry weight) per liter within 3 to 5 weeks incubation. It is clear, therefore, that the minimal growth require-

ments of tubercle bacilli are extremely simple.

These results have been obtained by inoculating synthetic media with large inocula, containing many billions of cells. In contrast, it is difficult or impossible to initiate the growth of small inocula in the same media. This is true with old laboratory strains and even more so on primary isolation from pathologic material. On the other hand, growth of small inocula, of a single cell under optimal conditions, has been obtained by utilizing a variety of media containing complex organic materials, and in particular egg yolk. It is for this reason that egg media are usually used for diagnostic work. The commonly accepted view is that these organic materials supply some growth factors which tubercle bacilli can synthesize only slowly and with great difficulty in synthetic media. There is increasing evidence, however, that failure of development of the

lipids and of other surface active agents are probably the most common offenders.

In fact, it has been recently established that the growth-inhibitory effect of many toxic agents, and particularly of long-chain fatty acids, can be neutralized by the addition to the medium of adequate concentrations of serum albumin. Indeed, far from being inhibitory, certain fatty acids can, under these conditions, exert a marked stimulatory effect on the growth of many, if not all, strains of tubercle bacilli. These observations have led to the development of new types of culture media (Dubos and Middlebrook, 1947) which permit more rapid growth of pathogenic mycobacteria and facilitate their isolation from pathologic material. Although there is not at the time of writing sufficient information to establish whether these new media will find a permanent place in diagnostic work, it may be justified to give here the composition of one of them.

#### OLEIC ACID-ALBUMIN MEDIUM

KH <sub>2</sub> PO <sub>4</sub> .....	1.0	Gm.	} Heat in 100 cc. distilled water to dissolve
Na <sub>2</sub> HPO <sub>4</sub> , 12H <sub>2</sub> O.....	0.3	Gm.	
Asparagine.....	2.0	Gm.	
ADD:			
Distilled water.....	850	cc.	
Enzymatic digest of casein.....	0.5	Gm. (10 cc. of a 5% stock autoclaved solution in distilled water)	
Ferric ammonium citrate.....	0.05	Gm. (1 cc. of a 5% stock solution in distilled water)	
MgSO <sub>4</sub> , 7H <sub>2</sub> O..	0.01	Gm. (1 cc. of a 1% stock solution in distilled water)	
CaCl <sub>2</sub> .....	0.0005	Gm. (1 cc. of a 0.05% stock solution in distilled water)	
ZnSO <sub>4</sub> ..	0.0001	Gm. (1 cc. of a 0.01% stock solution in distilled water)	
CuSO <sub>4</sub> .....	0.0001	Gm. (1 cc. of a 0.01% stock solution in distilled water)	
Adjust pH to 6.8			
Autoclave			
ADD:			
100 cc. of the following preparation of oleic acid-albumin complex:			
(1) Dissolve 0.12 cc. of oleic acid (0.1 Gm.) in 10 cc. of N/20 NaOH by shaking with a rotary motion in a small flask.			
(2) Add 5 cc. of this solution to 95 cc. of a neutral 5 per cent solution of serum Fraction V (albumin) in 0.85 per cent saline.			
(3) Sterilize by filtration through bacteriological filters, preferably glass or porcelain filters.			

small inocula is not always due only to nutritional deficiencies of the synthetic media, but also to the presence in the latter of substances which exert an inhibitory effect on growth and which exist as impurities in the reagents or on the glassware. Traces of toxic

In all the classic synthetic and egg yolk media, most strains of tubercle bacilli give a characteristic mode of growth consisting of large clumps, pellicles or heaped masses, probably resulting from the hydrophobic character of the bacterial surface. For sev-



eral reasons, not to be discussed here, this mode of growth gives rise to technical difficulties in diagnostic and experimental work. Fortunately, certain water-soluble esters of long-chain fatty acids are capable of wetting the surface of tubercle bacilli and of facilitating their growth in liquid media in the dispersed state. One of these esters, commercially known under the name of "Tween 80," contains oleic acid in a water soluble nontoxic form, available for metabolic utilization by the bacilli, and constitutes, therefore, a valuable adjuvant to culture media when dispersed growth is desired.

None of the known biochemical activities of tubercle bacilli are sufficiently characteristic to be useful in defining the group. All degrees of variation exist between the various types. All saprophytic strains of mycobacteria grow well on media adequate for the pathogenic strains but the growth of the former is in general so much more rapid that they can usually be distinguished by this character alone; moreover, most saprophytic strains give rise to much more pigmentation (yellow or orange) than the parasitic forms and are less acid fast than virulent human, bovine and avian tubercle bacilli. Saprophytic mycobacteria usually grow readily on ordinary broth agar without glycerine.

#### RESISTANCE TO PHYSICAL AND CHEMICAL AGENTS

Tubercle bacilli are as susceptible to desiccation, heat, radiation and physical agents in general as other nonsporulating bacteria. On the other hand, they are usually regarded as possessing unusual resistance to antiseptics and chemotherapeutic agents. Thus, the fact that many tubercle bacilli survive exposure to acids and alkalis for prolonged periods of time is made use of in the isolation of these organisms from pathologic material. It must be emphasized, however, that many of the tubercle bacilli die during this treatment and that, on the other

hand, many other bacterial species, in particular Gram-positive cocci, can survive and appear in the cultures unless inhibited by more specific means.

It is probable that tubercle bacilli owe part of their resistance to certain toxic agents to the hydrophobic character of their cell surface. In this respect, it is of interest that they are extremely susceptible to the bactericidal effect of surface active agents (anionic and cationic, natural and synthetic) which can become adsorbed through their own lipophilic chains onto the cell surface. Moreover, it has been recently observed that Tween 80 increases the susceptibility of tubercle bacilli to a number of antibacterial agents, to penicillin in particular, probably by wetting the bacterial surface (Kirby et al., 1947). It must also be recognized that the conclusion that mycobacteria are highly resistant to the bactericidal effects of antiseptics has been derived from tests usually carried out with suspensions consisting of large clumps of bacilli; it is probable that the organisms present in the center of these clumps are protected from contact with the bactericidal substance. In fact, the apparent insusceptibility is decreased when the tests are carried out with finely dispersed bacterial suspensions. It seems, therefore, that the resistance of tubercle bacilli to toxic agents has been somewhat exaggerated. Nevertheless, in practice, it is possible to find conditions under which tubercle bacilli are unaffected by concentrations of antibacterial agents which are bacteriostatic (or bactericidal) for other species. Thus, it is possible to add to culture media used in diagnostic work concentrations of penicillin or of malachite green which do not inhibit the growth of mycobacteria but which prevent the multiplication of Gram-positive cocci which have resisted treatment with acid or alkali.

Disinfection of sputa and excreta bearing tubercle bacilli can be effected by exposure to 5 per cent phenol or 2 per cent cresol solution for 12 hours. Tubercle bacilli can

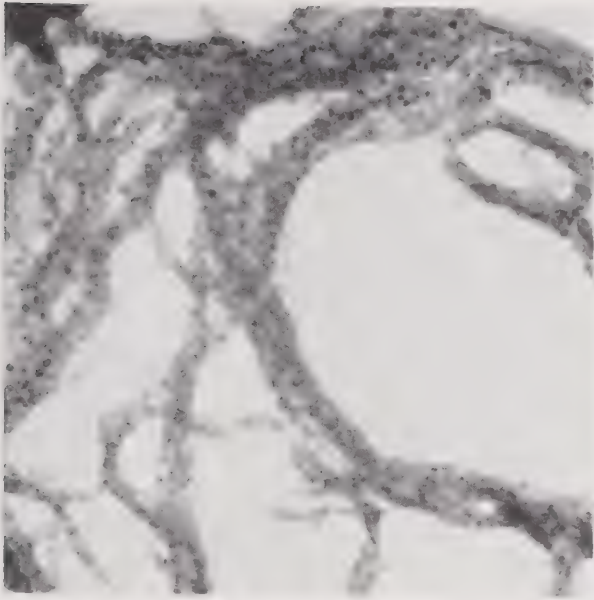


FIG. 11. Ziehl-Neelsen stained smear of a 7-day-old culture of a typical virulent human strain of tubercle bacilli (H37Rv) in a liquid medium containing oleic acid-albumin complex. Note the formation of serpentine cords.  $\times 1,065$ .

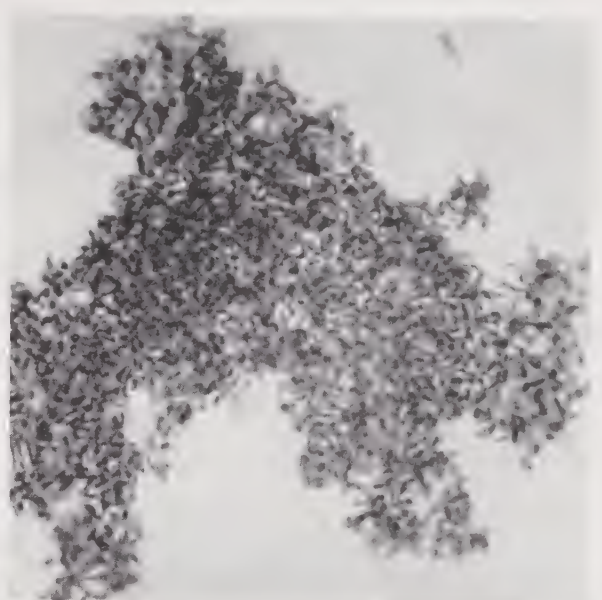


FIG. 12. Ziehl-Neelsen stained smear of a 7-day-old culture of a typical avirulent variant human strain of tubercle bacilli (H37Ra) in a liquid medium containing oleic acid-albumin complex. Note the lack of orientation in the arrangement of the bacterial cells in this clump.  $\times 1,065$ .

survive for long periods of time in the dried state in sputa and excreta.

#### VARIATION

It has long been recognized that cultures of virulent tubercle bacilli often become attenuated on prolonged cultivation on artificial media, particularly on media of acid reaction (pH 6.0) (Petroff et al., 1929; Smithburn, 1937; Oatway and Steenken, 1937). Although the unstable physical and chemical characteristics of solid egg media have led to much confusion in description and terminology, variation in the morphologic characteristics of cultures of mammalian tubercle bacilli were early observed to be correlated with variations in virulence (Petroff et al., 1929). Recent studies (Middlebrook et al., 1947) on the morphologic characteristics of tubercle bacilli in liquid and on solid media of relatively stable and reproducible composition (see Cultivation) have clarified and extended the earlier observations. Thus, it has been shown that all

virulent eugonic \* strains of human and bovine tubercle bacilli † tend to form microscopic serpentine cords of varying thickness and length consisting of strongly acid-fast bacilli oriented in parallel along the cords. The formation of cords appears to be an important factor in conditioning the ability of virulent cultures to spread on the surface of liquid and of solid media. Avirulent variants of virulent strains of mammalian tubercle bacilli, on the other hand, grow in a more or less nonoriented fashion; they do not form cords and the individual bacilli are less acid fast than those of virulent strains (Figs. 11, 12, 13 and 14). Certain strains of tubercle bacilli possess relatively stable morphologic and virulence characteristics which are intermediate between those of the fully

\* See section on characteristics of the different types of *M. tuberculosis*.

† All such cultures on primary isolation have a remarkably uniform high virulence for the guinea pig, except cultures from cases of lupus vulgaris (tuberculosis of the skin) which, peculiarly, have in many instances diminished virulence.



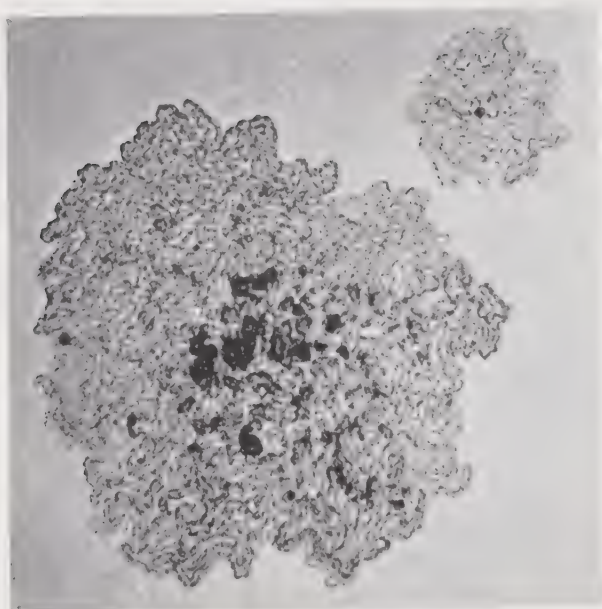


FIG. 13. Twelve-day-old culture of H37Rv on the surface of a solid agar medium containing oleic acid-albumin complex. The colonies are flat, spreading and translucent, consisting of serpentine cords.  $\times 85$ .

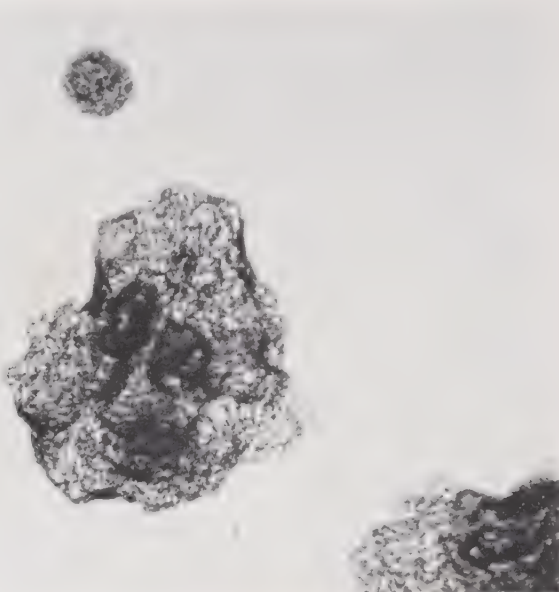


FIG. 14. Twelve-day-old culture of H37Ra on the surface of a solid agar medium containing oleic acid-albumin complex. These colonies, showing no evidence of cord formation, are heaped up and have little tendency to spread out over the surface of the medium.  $\times 85$ .

virulent and "completely" avirulent variants. The best-known strain of this type is BCG—bacillus of Calmette and Guérin (Calmette, 1936). This strain is an attenuated variant of an originally virulent bovine strain which was cultivated for many years on a glycerine-potato-bile medium. It has been extensively used for immunization purposes in man and is included in most studies on dissociation of tubercle bacilli. Although there is no authenticated case of progressive and fatal tuberculosis in human beings attributable to BCG, several groups of investigators claimed in the early years of its use that it could dissociate back to a virulent variant (Reed et al., 1932; Sasano and Medlar, 1931). There is little doubt that its virulence is not absolutely constant (Jensen et al., 1946) and that, indeed, a low but definite degree of virulence is desirable in cultures of BCG to be used for purposes of immunization, but dissociation to a fully virulent variant has not been observed in more recent years. The relative stability of the low virulence of this strain in contrast

to many other attenuated strains suggests that stepwise attenuation, analogous to mutation, occurs in tubercle bacilli as has been demonstrated in other bacteria.

No clearly defined structural or immunochemical differences in the bacterial cells have been correlated with the morphologic differences between cultures of tubercle bacilli of different degrees of virulence.

#### CHARACTERISTICS OF THE DIFFERENT TYPES OF *M. tuberculosis*

Table 31 summarizes the known degrees of pathogenicity of the three principal types of tubercle bacilli for various species of animals. Infection tests in the rabbit and the guinea pig will usually suffice to determine the type of an unknown culture. The human type has high pathogenicity for the guinea pig and low pathogenicity for the rabbit. The avian type has high pathogenicity for the rabbit in addition to pathogenicity for fowl, usually producing in the rabbit the

TABLE 31. PATHOGENICITY OF TYPES OF *M. tuberculosis*

ANIMAL SPECIES	TYPES OF TUBERCLE BACILLI		
	BOVINE	HUMAN	AVIAN
Guinea pig.....	++++	++++	0
Rabbit.....	++++	+	++++
Mouse *.....	++++	++++	+(?)
Hamster.....	++	+++	+
Anthropoids and monkey.....	++++	++++	0
Goat.....	+++	0	0
Horse.....	++	0	++
Dog.....	++	+	0
Cat.....	+++	+	0
Cattle.....	++++	0	0
Swine.....	+++	+	+++
Parrot, cockatoo..	+++	++	+++
Domestic fowl....	0	0	++++

"O" indicates that natural infection occurs most rarely although temporarily progressive lesions may be produced by the injection of relatively large numbers of living bacilli.

\* Certain strains of mice are highly susceptible to bovine and human types; others are very resistant (Pierce et al., 1947).

so-called Yersin type\* of disease, and has very low pathogenicity for the guinea pig. A fully virulent bovine culture has high pathogenicity both for the guinea pig and for the rabbit.† Strains of bovine tubercle bacilli which are somewhat attenuated may have high pathogenicity for the guinea pig but low pathogenicity for the rabbit and can, therefore, be confused with human strains.

Wells has isolated many strains of acid-fast bacilli from voles which commonly suffer from natural tuberculosis. These strains differ in certain respects from the other types of tubercle bacilli, sufficiently so that they are referred to as the murine type of

tubercle bacillus or *M. muris*. This type has low pathogenicity for the guinea pig and the rabbit, but is said to be pathogenic for the mouse (Wells, 1946).

Mammalian strains of tubercle bacilli (human and bovine) can usually be distinguished on primary isolation by cultural characteristics. Bovine strains grow poorly on the usual solid egg media and are inhibited by high concentrations of glycerine (more than 0.75 per cent); such cultures have been called "dysgonic." Human type strains, on the other hand, usually grow more luxuriantly on the solid egg media and are not so much inhibited by the presence of glycerine in the medium; indeed, their growth appears to be favored by glycerine, at least as far as the potential amount of growth is concerned; they are termed "eugonic." Dysgonic cultures present small, smooth, flat or hemispherical colonies with even, entire edges on egg media. Eugonic cultures grow in the form of rougher, usually more spreading colonies with thin, uneven edges on egg media. However, some dysgonic cultures have human type pathogenicity, and such distinctions between bovine and human cultures are not reliable, therefore, for conclusive type determination. Many dysgonic bovine cultures after repeated transfers on artificial media assume the eugonic characteristics of human cultures without change in pathogenicity or virulence (Frimodt-Möller, 1939). This change is apparently associated with loss of the ability to induce the formation of antibodies against a bovine type specific antigen (Schaefer, 1940) which is probably protein in nature.

Avian-type bacilli on primary isolation from diseased fowl or swine grow readily in the form of smooth hemispherical colonies on egg media. They tend to grow somewhat more dispersed than mammalian strains in the depth of liquid media. Dissociation of avian cultures to variants similar in colony morphology to those of human strains has been described. These changes may or may

\* The intravenous injection of large numbers of avian tubercle bacilli into the rabbit causes a rapidly fatal septicemia with the formation of tubercles of microscopic size.

† R. Koch maintained that there is only one type of mammalian *M. tuberculosis*. The credit for distinguishing the human and bovine types is due to Theobald Smith (1898).



not be associated with a change in virulence, but they are never accompanied by a change in pathogenicity; that is, such cultures never become pathogenic for the guinea pig (Feldman, 1938).

Avian strains of tubercle bacilli are distinguishable from the mammalian strains by serologic methods (Furth, 1926). The former possess an antigen or antigens which are absent in the latter. Avian strain tuberculin is also distinguishable from mammalian strain tuberculins by quantitative skin tests. Human and bovine strains, on the other hand, are not distinguishable from each other by routine serologic methods and their tuberculins are so similar as to be considered identical in skin tests.

Murine strains grow more slowly on egg media than do other tubercle bacilli; their growth is inhibited by glycerine. They are not distinguishable serologically from the human and bovine strains (Wells, 1946).

#### PATHOGENIC PROPERTIES OF THE DIFFERENT TYPES FOR MAN

The human and bovine types are the principal agents of tuberculosis in man. The human type is usually found in cases of pulmonary tuberculosis. In countries where bovine tuberculosis is not uncommon the bovine type is often the agent of bone and joint tuberculosis and tuberculous cervical lymphadenitis which occur most often in children. This apparent difference in organ specificity of the two principal mammalian types is due to the fact that the route of primary infection determines in large measure the predominant organ pattern of tuberculous disease. Infection by inhalation tends to produce pulmonary disease, whereas primary infection by the gastro-intestinal tract as from ingestion of unpasteurized milk containing bovine type tubercle bacilli tends to result in the extrapulmonary patterns of disease. In rural areas (few in the United States) where cattle tuberculosis is widespread, the bovine type is not an uncommon

agent of pulmonary tuberculosis. The human type is not more pathogenic for man than the bovine type.

Although there have been described a few well-authenticated cases of progressive tuberculosis in man due to the avian type of tubercle bacillus (Feldman, 1938; Bradbury, 1946), they are very rare.

#### CHEMICAL CONSTITUENTS OF MYCOBACTERIA

The relationships between chemical constituents and pathogenicity or virulence of mycobacteria are not known. It is well established that the chemical constituents of these organisms can vary qualitatively and quantitatively, depending on the method of cultivation. Nevertheless, the following information is available as to the chemical constituents of mycobacteria and their relationship to the immunology and pathology of tuberculosis (Wells and Long, 1932).

Each type of tubercle bacillus contains several protein fractions. The proteins are of special significance because they elicit the "tuberculin" reaction. It appears that all of the protein fractions of any particular type of tubercle bacillus can evoke the tuberculin reaction in an animal such as the guinea pig sensitized with the homologous type. It is unlikely that a nonprotein substance contaminating the protein fractions is responsible for the tuberculin reaction and no information is available as to whether one or several specific polypeptide or protein configurations are responsible for tuberculin activity. Certain protein fractions isolated from tubercle bacilli (and other mycobacteria) can induce the formation of precipitins, agglutinins and complement fixing antibodies as well as anaphylactic sensitization. The repeated injection of these proteins into normal animals does not induce the delayed type of reaction characteristic of tuberculin sensitivity. The proteins of human and bovine types cannot be easily distinguished from one another by serologic means or by skin sensitization (see Varia-

tion). The proteins of avian tubercle bacilli, Johne's bacillus, and saprophytic mycobacteria can readily be distinguished from those of mammalian tubercle bacilli by serologic means and by quantitative skin tests in hypersensitive individuals, but there are cross reactions throughout the genus *Mycobacterium*. This cross reactivity does not interfere with the practical usefulness of the tuberculin test in man (see Tuberculin). The P.P.D. (purified protein derivative) of Seibert (Seibert et al., 1934) is a standard preparation of tuberculin containing a mixture of tuberculoproteins, obtained by repeated trichloroacetic acid precipitation of the culture filtrates of tubercle bacilli. Extensive studies have been made on the separation and purification of the proteins of mycobacteria (Seibert, 1941).

Tubercle bacilli contain serologically active and inactive polysaccharides of high molecular weight, including glycogen (Muel-ler, 1926; Heidelberger et al., 1937). The mammalian tubercle bacilli appear to contain at least two serologically active polysaccharides. They can be distinguished to some extent from the polysaccharides of the avian and of other types of mycobacteria. Tuberculous hosts may become hypersensitive to homologous polysaccharides; this sensitization is of the anaphylactic type—distinct from the tuberculin type (Enders, 1929) (see Allergy). The roles of the polysaccharides of tubercle bacilli in the pathogenesis of tuberculosis have not been established.

The mycobacteria have long been known to be very rich in lipids. The systematic investigations of Anderson and his associates (Anderson, 1939-1940) have revealed a great variety of complex lipids, fatty acids, waxes and higher alcohols. The lipids are probably bound to proteins and polysaccharides in the bacterial cell, some more firmly than others. Many of the lipids are not easily destroyed in the animal tissues and are probably responsible for certain aspects of the cellular reactions to tubercle bacilli (Sabin,

1941). A cellular response resembling the tubercle (see Lesions Caused by the Tubercle Bacillus) can be produced by phosphatide fractions isolated from tubercle bacilli grown in vitro. Certain other lipid fractions obtained from tubercle bacilli or saprophytic mycobacteria or from unrelated sources provoke somewhat similar reactions. However, even when lipids of tubercle bacilli are used, an enormous quantity is required to produce tuberclelike lesions or caseation necrosis. There are chemical differences between the lipids extractable from different types of mycobacteria (mammalian, avian, saprophytic) cultivated on the same medium, and they vary with changes in the chemical composition of the medium. The possible role of the complex lipids in the staining properties of mycobacteria has not been clearly established, but it is known that treatment of tubercle bacilli with 1 per cent hydrochloric acid in ether-alcohol solution renders the cells non-acid fast and at the same time allows extraction of certain firmly bound lipids which are otherwise not extractable from the cells with neutral fat solvents such as ether and chloroform. There is no doubt, however, that acid-fastness is not due to the mere presence of lipids alone.

## TUBERCULOSIS

The host-parasite relationship is of such a nature in tuberculosis that the immunology and the pathology of the disease cannot be discussed separately. During the course of the infection, the character of the cellular response of the host changes, and the parasites find modified conditions for their multiplication and dissemination. In many other infections, specific immunology can be studied in terms of the relationship between specific antigenic components of the parasitic cells and protective humoral antibodies of the host. In tuberculosis, however, this relationship is obscure. In this



disease, acquired specific resistance, i.e., immunity, in the present state of our knowledge can be approached only by the description of events during the course of infection as seen in the morphologic pathology. Therefore, the pathologic aspects of tuberculosis will be emphasized here.

#### PATHOGENESIS

There are few infectious diseases in which it is known that the action of a single factor is of paramount importance in the host-parasite relationship (Dubos, 1945). The pathogenic action of certain micro-organisms is referable to the toxins which they produce. For example, the diphtheria bacillus produces an exotoxin which acts both locally at the site of its production and, after its absorption, upon various distant organs. Most manifestations of diphtheria can be reproduced by the toxin alone; moreover, recovery from diphtheria depends on neutralization of toxin by the specific antitoxin. In some infections the multiplication of bacteria in the host or the virulence of the micro-organism may be related to the presence or absence of a single, specific constituent of the bacterial cell such as a capsular substance. An example of this type is the pneumococcus. As yet there is no evidence indicating that the pathogenic properties of virulent mycobacteria depend on a toxin or on some particular constituent of the bacterial cell present in the virulent and absent or reduced in amount in avirulent strains. The main biologic difference between the pathogenic and nonpathogenic mycobacteria seems to be their relative capacities to multiply in a host. The nonpathogenic mycobacteria, such as *M. butyricum*, *M. phlei* and others, are true saprophytes multiplying outside of animal hosts and are not able to infect animals (except perhaps in rare instances).

As mentioned above, none of the constituents of the tubercle bacillus are known to be

especially toxic for normal animals.\* During the infection, however, the protein fractions become injurious to the host, i.e., the animal becomes hypersensitive to these antigens. Concomitant with the development of allergy is the development of a relative immunity and the formation of humoral antibodies against certain of the protein and carbohydrate antigens present in the bacterial cells. The immune response of the host can be duplicated by introducing killed tubercle bacilli into the normal animal. The process of immunity, inclusive of allergy, modifies disease and probably is an inseparable part of the tuberculous infection. Pulmonary phthisis as generally seen in adults is conditioned by the partial immunity resulting from previous infection during childhood.

In some of the typical acute infectious diseases due to bacteria, the infectious agents act on a host which has had no previous effective contact with the agent, or if it had suffered a prior infection, the immunity is lost before the new infection sets in. In such acute diseases, lobar pneumonia or diphtheria or typhoid, for example, the agent multiplies until the host dies or recovers. The recovery is due to an immune process and, as a rule, the infectious agent is destroyed completely. In tuberculosis, however, the infectious agent at first multiplies unopposed and later, due to the development of some immunity, the multiplication becomes partially restricted, but the causative agent usually is not completely destroyed. Indeed, a few viable tubercle bacilli may survive in the lesion and may, even after years, reinfect the host. Thus there is an equilibrium between the host and parasite which may shift in favor of the host or

\* This does not necessarily mean that tubercle bacilli as they multiply in a susceptible host do not produce a substance or substances possessing primary toxicity. Indeed, some evidence of primary toxicity is found in the fact that tuberculosis associated with marked clinical symptoms and high fever frequently responds to chemotherapy (streptomycin) with remarkable cessation of symptoms within 72 hours (McDermott et al., 1947).

the parasite. It is characteristic for this infection that in the same organ, healing and progressing lesions may coexist. For example, in a single section of tuberculous lung, it is often possible to observe some lesions which appear to be healing with fibrosis and others which seem to be progressing. Thus, it appears that in every focus of infection a process goes on, influenced in part by systemic and in part by local factors.

#### LESIONS CAUSED BY THE TUBERCLE BACILLUS

The tubercle bacillus produces in general two types of lesions. One type is called exudative, the other, productive or proliferative. The productive type is classified as an infectious granuloma since it resembles both granulation tissue and a tumor.

By examining the lungs of rabbits at different intervals of time after intravenous injection of living, virulent bovine type tubercle bacilli, one can observe that during the first day there is acute inflammation about the bacilli. There is exudation of fluid and accumulation of polymorphonuclear leukocytes, some of which contain bacilli. During the succeeding few days the exudation of fluid diminishes, and the polymorphonuclear leukocytes begin to die; monocytes appear, phagocytizing both the dead leukocytes and the tubercle bacilli. This exudative phase resembles pneumonia caused by pyogenic bacteria in some respects at least: namely, there is an exudate in the alveolar spaces and the elements of the normal tissue, lung, for instance, are included. The exudative lesion, actually seen most often in the lung, may be microscopic in size or may involve an entire lobe. Its size and the rate and violence with which it develops are functions primarily of two variables, the numbers of tubercle bacilli involved and the susceptibility of the host. In the normal tuberculin negative host, the exudation of fluid tends to be much less marked and the cellular response less vig-

orous. In the hypersensitive (allergic) host the lesion is a very acute and labile type of response seen most often when large numbers of bacilli reach an undiseased part of the lung by aspiration from another part containing a cavity. The exudative type of lesion may have a number of possible fates. It may heal by resolution; that is, the multiplication of the bacilli is inhibited and they may be destroyed, and tissue healing may take place without scar. It may undergo early necrosis, sometimes involving a whole lobe, and lead to massive cavity formation. Or, it may develop into the productive type of lesion, the most frequently observed lesion in tuberculosis because of its chronic character. This lesion presumably develops from the exudative type in the following manner. After the diminution in exudate and appearance of mononuclear phagocytes of the exudative lesion, cells of a different type, epithelioid cells, appear and increase in number. This cell has a pale cytoplasm and a large, elongated nucleus. The faintly outlined epithelioid cells are of irregular form and are usually arranged side by side. At this time, the acid-fast bacilli can be found in these cells, but it is noteworthy that only a few of them contain stainable bacilli. After two or three weeks, if the lesion does not spread too rapidly, a zone of proliferating fibroblasts mingled with lymphocytes appears at the periphery, and in the center of the older lesions giant cells may be found. These cells are characterized by their large size, being up to several hundred micra in diameter, and by their numerous, dark-staining nuclei, situated on the periphery of the cytoplasm; these cells also may contain stainable tubercle bacilli. The typical lesion now has three zones, a central giant cell or zone of giant cells, a midzone of epithelioid cells arranged radially and a peripheral zone of fibroblasts, lymphocytes, monocytes, and plasma cells supported by a newly formed reticulum.

The cellular response just described is



characteristic of the productive type of tuberculous lesion and is defined as the microscopic tubercle. In human disease, it is seen macroscopically as a barely visible grayish, translucent nodule; it may become larger, opaque and yellowish as necrosis occurs in

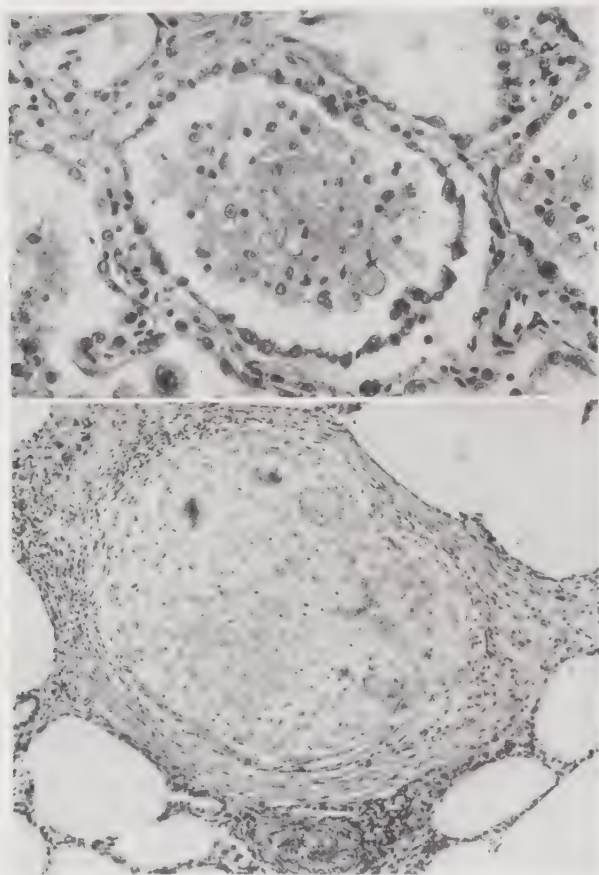


FIG. 15. (*Top*) Exudative type of lesion. Acute tuberculous pneumonia. Edema of alveolar septae and exudate with mononuclear phagocytes in alveolar spaces. (Pinner, M., 1945, *Pulmonary Tuberculosis in the Adult*. Springfield, Ill., Thomas.)

FIG. 16. (*Bottom*) Proliferative or productive type of lesion. Epithelioid tubercle of the lung showing epithelioid cells, giant cells, fibrous capsule and a few lymphocytes at the periphery. (Pinner, M., 1945, *Pulmonary Tuberculosis in the Adult*. Springfield, Ill., Thomas.)

the center of the lesion. Such a macroscopic tubercle is often a coalesced group of microscopic tubercles. Thus, the tubercle may grow in size by extension or by fusion with other tubercles and become necrotic. The

necrotic material has the consistency and appearance of cheese; the process is, therefore, called caseation necrosis. The biochemical factors in the production of this peculiar type of necrosis are not clear, but it is believed that certain autolytic enzymes of the host, which normally effect liquefaction to pus, are inhibited. A caseous tubercle, small or large, may break into a bronchiolus or bronchus and empty its contents; this results in cavity formation. On the other hand, calcium salts may be deposited in the caseous material of the walled off tubercle. The older tubercle may become surrounded by a thick layer of fibrocytes in the form of a capsule, and fibrous tissue with vascularization may penetrate and replace the tubercle. Calcification and even ossification of healed pulmonary tubercles occur frequently in childhood.

Of primary importance, it appears, for the fate of the early exudative lesion are: the initial number of infective tubercle bacilli, their rate of multiplication in the particular host, and the degree of hypersensitivity of the host. A small number of infecting bacilli with little or no multiplication presumably leads to resolution and disappearance of the lesion, sometimes without typical tubercle formation. A large initial number of bacilli, or their rapid multiplication (in a host with low resistance), or a high degree of hypersensitivity of the host, lead to early and widespread necrosis, even in areas where, presumably, the typical tubercle never has an opportunity to form (caseous and gelatinous pneumonia). A small initial number of bacilli with slow multiplication, continuous or discontinuous, results in the proliferative (or productive) type of lesion.

The accompanying illustrations (Figs. 15 and 16) show the principal characteristics of the two predominant types of tuberculous lesions. It must be remembered that both the productive and the exudative types of lesions are usually present in chronic progressive tuberculosis in man; they may

be anatomically contiguous and lesions intermediate between the two types are frequently evident (Pinner, 1945).

#### SPREAD OF TUBERCLE BACILLI IN THE HOST

Although tubercle bacilli multiply readily in artificial media in the absence of tissue cells, they are seen to be primarily intracellular in the infected host. They seem to multiply intracellularly. Nevertheless, they do occur also extracellularly, and an extracellular phase has to be assumed in the progressive spreading infection.

Tubercle bacilli may spread by contiguity, by lymphatic drainage, by the blood stream, and by "tubular" means.

Tubercle bacilli, like other microscopic particulate matter present in organs, usually reach the nearest regional lymph node by *lymphatic dissemination*. There, multiplication may take place and the bacterial cells may pass to other lymph nodes. Thus, a chain of lymph nodes may be infected, and the tubercle bacilli may find their way through the thoracic duct into the blood stream resulting in systemic dissemination and miliary tuberculosis. When a host is infected for the first time (regardless of whether the portal of entry is pulmonary or intestinal) the draining lymph nodes almost always develop tuberculous lesions. For this reason the tuberculous foci of the hilar lymph nodes were called by Parrot (1876) "*miroirs des poumons*." Tuberculous infection contracted by ingestion results in the involvement of mesenteric lymph nodes. While the infection of the draining lymph nodes occurs with the regularity of a biologic law during the first infection, lymphatic spread is almost always absent or inconspicuous during reinfection.

*Hematogenous dissemination* of tubercle bacilli occurs most frequently during the progress of first infection tuberculosis and originates, as a rule, either in a caseous mediastinal lymph node or by the invasion of a vein by a growing tubercle. The uni-

form distribution of tubercles in the lung as seen by X-ray may allow a diagnosis at this stage. Rarely, tubercle bacilli may be cultured from the blood. Tuberculous meningitis, a result of bacteremia, occurs more frequently in children following first infection than in adults who have miliary tuberculosis.

The result of hematogenous distribution, i.e., the development of tubercles, seems to depend on the susceptibility of various organs. The thyroid gland, the pancreas, the heart and voluntary muscles rarely or almost never are the sites of miliary, blood-borne, tubercles. In parts of the lung compressed because of effusion or pneumothorax there may be fewer and smaller tubercles than elsewhere in the lung. This, however, may be due merely to diminished blood supply.

It may be noted that parts of the lungs may show fairly evenly distributed tubercles suggesting hematogenous dissemination because of their distribution and uniformity as to size and character. This hematogenous miliary tuberculosis may not be associated with recognizable tubercles in other susceptible organs, such as the spleen, liver or kidneys. Following hematogenous dissemination, the tubercles in the lungs may be exudative or productive or the central part exudative surrounded by a productive zone. In other organs they are, as a rule, of the productive type. Hematogenous spread may occur repeatedly.

Tuberculosis of the kidney, liver, spleen, bone, testes, ovaries, and other organs is a consequence of blood invasion. Occasionally, at necropsy of persons dying of causes other than tuberculosis, minute tuberculous lesions, mostly calcified, are found in the kidneys, liver, or spleen.

In *tubular dissemination*, the contents of a discharging cavity reach the bronchi with subsequent aspiration into the parenchyma of the lung. Tuberculous laryngitis, tonsillitis and enteritis are the results of such a spread. Similarly, tuberculosis of the kidney



may lead to tuberculous cystitis. The tubular spread of tubercle bacilli plays a conspicuous part in adult pulmonary tuberculosis. For example, a cavity in the upper lobe discharges its contents into a bronchus. Particularly if the discharge material is liquid, it may be aspirated into other portions of the same or opposite lung and there initiate new foci of disease. Tuberculous caseous bronchopneumonia is apt to occur following hemoptysis and aspiration of blood containing viable tubercle bacilli.

#### TUBERCULOSIS OF FIRST INFECTION OR OF CHILDHOOD TYPE AND TUBERCULOSIS OF REINFECTION OR OF ADULT TYPE

In the United States tuberculosis of first infection is almost always the result of inhalation of the human type of tubercle bacilli. In countries where unpasteurized milk contaminated with bovine tubercle bacilli is consumed, the primary infection arises in the intestines in an appreciable number of cases.

Tuberculosis of the reinfection type, or adult type, designates tuberculosis in an individual who has passed through first infection, i.e., who has a healed primary lesion (not necessarily in a bacteriologic sense, because sometimes the healed lesion of the first infection contains viable tubercle bacilli). The reinfection may be the exacerbation of the first infection or, more commonly, a newly acquired infection.

An illuminating analogy of the childhood and adult types of tuberculosis can be recognized in experimental animals (see Immunity-Koch phenomenon). When tubercle bacilli are deposited in the skin or subcutaneous tissue of a guinea pig, the draining lymph nodes are soon involved and progressive disease follows. If the guinea pig is infected subsequently in the same way again but at another site, on the opposite leg, for instance, the tubercle bacilli remain entirely or almost entirely localized; they may multiply locally but do not, or only in small

numbers, invade the draining lymph node. The prevention of lymphatic spread is concomitant with the development of hypersensitivity to tuberculin.

Pulmonary tuberculosis of first infection differs in many respects from that of reinfection or adult type (Ghon, 1916; Opie, 1917). Whereas the location of the lesion of the first infection may be in any part of the lung and in the apex not more frequently than elsewhere, the lesion of the reinfection tuberculosis almost always has its origin near the apex. A conspicuous involvement of a hilar lymph node almost always follows the lesion of the first infection but practically never follows the lesion of reinfection. In the childhood type of tuberculosis, an inconspicuous parenchymal lesion often leads to massive caseation of the draining lymph node\* while in the adult type of tuberculosis the hilar lymph nodes practically never contain caseous lesions even if whole lobes are involved in the infection. The childhood type of tuberculosis is an acute type of disease healing or progressing in a relatively short time; the adult type, on the contrary, is more stable, more chronic. In the childhood type of tuberculosis there is little or no formation of fibrous tissue, and the disease may progress so rapidly that death occurs before cavities can develop. However, in the adult type of disease the progress is usually chronic; fibrosis is conspicuous and cavity formation is the rule. The disease usually begins in an upper lobe and spreads downwards, clearly through tubular routes.

There is an essential histologic difference between the primary and the reinfection type of lesion. The childhood type is almost always exudative, the reinfection type is at first productive but may lead to the exudative type, usually as a result of tubular spread. The childhood type often heals by resolution or undergoes caseation and calci-

\* The primary pulmonary complex, "Ghon complex," consists of the primary parenchymal lesion ("Ghon tubercle") and the involved regional, hilar lymphatic tissue.

fication. The reinfection type may caseate and lead to cavitation or may heal with scar formation, but healing by resolution can rarely be observed.

The synonyms, childhood and first infection types on the one hand and reinfection or adult types of tuberculosis on the other, originate from the epidemiologic observation that under the conditions which have been prevalent until now, most of the first infections occur in children. Actually the character of tuberculosis may not depend on the age of the host but rather on preceding immunologic status, i.e., the existence of a prior tuberculous infection. The childhood type of disease may be observed in adults who have escaped earlier infection. On the other hand, adults, acquiring their first infection, as indicated by previous tuberculin test, may develop the adult or reinfection type of disease. This occurs more often in persons who move from a rural to an urban environment. In the United States the rapidly progressive childhood type of tuberculosis is seen more often in young adult Negroes than in white adults.

The healing of first infection and the appearance of reinfection may be separated by an interval of many years. The question arises as to how the relative immunity is maintained. Do the tubercle bacilli in the healed lesion of the first infection survive and thus maintain the immunologic status of the host? Are they dead but not destroyed and eliminated? Careful studies of healed lesions of first infection have shown that in many cases there are no viable tubercle bacilli; on the other hand, epidemiologic data show that tuberculin sensitivity, once developed, is usually lasting. The explanation may be that casual contacts with tubercle bacilli are enough to maintain tuberculin sensitivity after infection has once taken place even if these chance contacts do not produce recognizable lesions. It may be added that first-infection tuberculosis has been observed occasionally at autopsy in persons whose lungs have sustained a healed

primary complex—healed parenchymal and hilar lymph node lesions (Terplan, 1940). It is possible that in these persons the complete healing of first infection was followed by loss of tuberculin sensitivity and complete reversal to the original immunologic status.

#### IMMUNITY

The term immunity is used here in a restricted sense, meaning specific, acquired resistance to infection.

In immunity against tuberculosis the role of *humoral antibodies* has not been demonstrated. Specific antibacterial sera agglutinate tubercle bacilli in vitro; they promote phagocytosis by polymorphonuclear leukocytes and monocytes in vitro; they fix complement in the presence of tubercle bacilli and certain of their antigenic components; they form precipitates with protein and carbohydrate fractions of tubercle bacilli. There is no evidence, however, that immune sera have bactericidal or lytic effects.

In many pathogenic species of bacteria, the virulent variants possess surface antigens which are absent in certain avirulent variants and acquired resistance is usually associated with antibodies against these antigens (Dubos, 1945). In the case of tuberculosis, no antigens related to virulence have been discovered, although morphologic differences between virulent and avirulent variants are conspicuous (see Variation).

Infection with tubercle bacilli enhances the ability of the host to respond to antigens not related to tubercle bacilli. Antibody formation is nonspecifically increased and sensitization is altered (Dienes et al., 1927). The addition of paraffin oil to killed tubercle bacilli increases and prolongs antibody formation and sensitization to the antigens of tubercle bacilli. The antigenic effect of certain other antigens incorporated in paraffin oil with killed tubercle bacilli is also greatly increased and prolonged (Freund, 1947).

In addition to the formation of humoral



antibodies, the tuberculous host develops another type of immune process, namely, an altered tissue reactivity to tubercle bacilli and their components, called *allergy*. Certain aspects of immunity in tuberculosis are well illustrated by the experiment known as the *Koch phenomenon*: when a guinea pig is injected in the subcutaneous tissue of the thigh with living, virulent tubercle bacilli, the puncture wound heals within two days. From 10 to 14 days later a nodule appears at the site of injection. It ulcerates, and the ulcer usually does not heal. The regional lymph nodes develop tubercles and caseate. In contrast to this, the already tuberculous guinea pig reacts differently to infection. When after a period of a few weeks the infected animal is again injected in the same way, in the opposite thigh, a dark-colored induration about 1 cm. in diameter develops within two days at the site of injection. The skin over the indurated area undergoes necrosis and soon a superficial ulcer appears. This ulcer, however, heals quickly. Infection of regional lymph nodes is retarded or fails to develop. The conclusion may be drawn that during the infection two changes have occurred: the host has become hypersensitive to tubercle bacilli and also has acquired the capacity to localize the superinfection. Acquired resistance to infection with highly virulent tubercle bacilli in the allergic animal can be best shown by infecting guinea pigs first with a strain of low and later with a strain of high virulence. The progress of superinfection is retarded or entirely inhibited.

Koch found that tuberculous guinea pigs react with inflammation not only to living but also to killed tubercle bacilli as well to their protein fractions (Tuberculin). Later it was established that the Koch phenomenon can be demonstrated in guinea pigs sensitized by killed as well as by living tubercle bacilli. Neither tuberculin nor any fraction of tubercle bacilli has been shown to induce tuberculin sensitization or the Koch phenomenon. It is possible, however,

that a combination of tuberculo-protein and a lipid fraction might do so; this possibility has been recently suggested. It would appear that the cells present in the tubercle play an essential role in the development of allergy to protein components of tubercle bacilli. Certain aspects of this subject are dealt with in the chapter on Allergy, particularly the differences between the tuberculin type and the other types of hypersensitiveness.

The Koch phenomenon is a useful paradigm of immunity experiments because it takes place in the skin and is accessible to direct observation. Essentially the same events occur, however, when the bacilli are introduced into the lung by inhalation or by the intravenous route.

For the understanding of the mechanism of immunity in tuberculosis, the fate of tubercle bacilli of reinfection and the cellular reaction to these organisms are of particular importance. One striking difference between the fate of tubercle bacilli in the unprepared and in the immune animal is the rate of lymphatic spread, as mentioned above. Krause (1926) injected tubercle bacilli into the skin of sensitized and normal guinea pigs. Early excision of the skin lesion prevented tuberculosis in the former but not in the latter animals. He felt the retardation of lymphatic spread was due to formation of a mechanical (fibrin) barrier. Does the inhibition of lymphatic dissemination depend on the prompt destruction of bacilli at their deposition? Cultural studies of the excised lesions have shown that at the site of their original deposition the tubercle bacilli may multiply considerably and yet their appearance in the regional lymph nodes is retarded (Freund and Angevine, 1938). Perhaps the specific inflammation at the site of injection may promote their local fixation even before gross signs of inflammation are demonstrable.

In addition to the retardation of the lymphatic dissemination in the immune animal, tubercle formation is accelerated and the newly introduced tubercle bacilli are de-

stroyed or, at least, their multiplication is retarded. At the site of the deposition of tubercle bacilli in the immune animal the accumulation of monocytes and the appearance of epithelioid cells is rapid; the cellular reaction is nodular. In the nonimmune animal the cellular response (mononuclear cells) is slow and the distribution of inflammatory cells is more diffuse. If the number of tubercle bacilli injected into the immune animal is not excessive, the tubercle heals with fibrosis; in the nonimmune animal it progresses and becomes caseous. The number of tubercle bacilli in the lesions can be estimated by staining sections and by cultural methods. As one would expect, the tubercle bacilli diminish, sometimes after brief preliminary multiplication, in the immune, and steadily increase in the non-immune animal. The tubercle bacilli at first may be seen in monocytes and later only in epithelioid cells and occasional giant cells. It appears as if they would multiply or be destroyed in the epithelioid cells.

By counting the colonies of tubercle bacilli obtained from weighed amounts of organs of infected rabbits Lurie (1942) reinvestigated the relationship of tubercle bacilli to the cellular reaction to them. His studies led him to conclude that the bacilli first multiply in the mononuclear phagocytes almost unopposed. Later, coincident with the appearance of hypersensitiveness to tuberculin and the first stages of caseation, the mononuclear cells become epithelioid cells and the numbers of bacilli diminish. In the resistant host both the formation of epithelioid cells and the destruction of bacilli are rapid. They are also more rapid in a more resistant organ, such as the liver, than in a less resistant organ, such as the lung, of the same host. In the immune rabbit in the presence of a primary lesion the bacilli of the reinfection are quickly destroyed without preliminary multiplication and with rapid formation of mature tubercles. Lurie also observed that mononuclear phagocytes obtained from the peritoneal cavities of tuberculous rabbits have an increased capacity to destroy tubercle bacilli as well as an increased nonspecific capacity for phagocytosis of particulate matter such as carbon particles.

The tubercle, its monocytes and epithelioid cells, and the accelerated formation of tubercles are generally viewed as the means of defense of the host against the parasite.

The significance of the role of allergy in immunity against tuberculosis is the subject of intense controversy which is discussed by Rich (1944). It is widely believed that tuberculous pneumonia, caseation and cavity formation are due to or promoted by sensitization because they do not occur before sensitization becomes demonstrable and they are inconspicuous in species of animals, in rats for instance, whose skin does not react to tuberculin. Even if sensitization does favor the occurrence of caseation, the fact remains that caseation is less conspicuous in allergic than in normal rabbits if infected with a moderate dose of tubercle bacilli. It has been shown that tuberculous pneumonia is resolved more readily in allergic rabbits.

Effort has been made to study the relationship of *allergy* to *immunity* by desensitizing allergic guinea pigs. The experiments are difficult to interpret and have not led to unequivocal conclusions.

As mentioned above, when the Koch phenomenon is elicited with large numbers of tubercle bacilli, the allergic animals respond with an intense inflammation which results in the necrosis and sloughing of the skin at the site of injection. The externalization—casting out of tubercle bacilli with the lesion—is of advantage to the host. When, however, the site of the reinfection is the lung, a necrosis and sloughing of the lesion may lead to the spread of bacilli through the bronchi (tubular spread).

The chance of contracting progressive, clinical tuberculosis when exposed to tuberculous infection is higher in tuberculin-negative than in tuberculin-positive persons (Ferguson, 1946). This has been observed repeatedly among student nurses and in other groups. Particularly convincing are the observations made in an insane asylum where the exposure was high and consider-



able numbers of inmates were tuberculin negative when admitted (Flahiff, 1939). Such observations in man and immunization experiments in animals have encouraged the study of prophylactic, active immunization of man.

#### IMMUNIZATION

In experimental animals, repeated injections of killed tubercle bacilli produce a moderate degree of immunity against infection with highly virulent organisms. This can be shown best in rabbits (Opie and Freund, 1937). Some of the immunized animals escape the disease; the majority develop tuberculosis with a course slower than in the unimmunized rabbits. The immunizing effect of heat-killed tubercle bacilli was studied in a hospital for the insane by inoculating alternate tuberculin-negative patients within two weeks after admission (Opie et al., 1939). The morbidity and mortality rates during a period of 18 months following vaccination were appreciably lower in the vaccinated than in the nonvaccinated group.

There is good evidence that in experimental animals BCG and other living attenuated strains are more effective than heat-killed tubercle bacilli.\* It has been shown by many groups of workers that vaccination with BCG affords considerable protection against infection (Holm, 1946). Aronson and Palmer (1946) vaccinated 1,550 tuberculin negative persons in Indian reservations. These persons and 1,457 controls were followed for 6 years with annual tuberculin tests and X-rays. In terms of death per 1,000 person-years, the tuberculosis death rates were 7.2 in the controls and 3.8 in the vaccinated group. Furthermore, the incidence of disease, clinically manifest or diagnosable only by X-ray, was lower in the vaccinated group.

\* This does not necessarily imply that tubercle bacilli multiplying in vivo produce a protective antigen which is not produced in vitro.

The vole bacillus, *M. muris*, has been shown capable of inducing an active immunity in the guinea pig against infection with virulent strains of the other two mammalian types. The degree of active immunity is comparable with that produced by BCG. Experimental immunization of human beings with vole bacilli is being undertaken with the belief that a spontaneous change in pathogenicity of this type of tubercle bacillus for man is less likely than a spontaneous increase in virulence of BCG or similar attenuated bovine or human strains (Wells, 1946).

The difficulties of preparing a vaccine with predictable numbers of living bacilli of fixed virulence and the complications which sometimes occur after vaccination (suppuration of lymph nodes) has, until recently, caused postponement of large scale immunization of man in the U.S.A.

Active immunization with whole tubercle bacilli—BCG, other attenuated strains, vole bacilli, or killed tubercle bacilli—may have usefulness in the protection of tuberculin-negative individuals who are exposed to tuberculosis. It should be recalled, however, that persons with naturally acquired reactivity to tuberculin or bearers of old and latent tuberculous lesions can acquire new infection and progressive clinical disease. It is unlikely that artificial immunization with the vaccines thus far offered is more effective than infection itself; thus, solid group immunity after these types of vaccination cannot be expected. Nonetheless, it is desirable to make available a prophylactic vaccine such as BCG where its use is indicated.

#### CHEMOTHERAPY

There are innumerable chemical agents which can exert a bacteriostatic effect on tubercle bacilli in vitro. These agents, with only 3 exceptions thus far, are too toxic to be considered as chemotherapeutic agents. The sulfones (derivatives of diaminodiphenylsulfone) and related sulfonamidelike

agents have some retarding effect on the progress of tuberculosis (Feldman, 1946; Smith, 1945; Middlebrook, 1945). These compounds are purely bacteriostatic in vivo. They do not cure tuberculous infections in animals even under the most favorable experimental conditions. Their toxicity, particularly for the hematopoietic system, and other characteristics have limited their clinical application.

Streptomycin, a product of *Streptomyces griseus* (Schatz and Waksman, 1944) has high and uniform bacteriostatic activity against all strains of freshly isolated mammalian tubercle bacilli in vitro; it is also bactericidal in somewhat higher concentration for multiplying but not for nonmultiplying tubercle bacilli (Middlebrook and Yegian, 1946). It is capable of retarding the progress of tuberculosis both in the experimental animal and in man (Hinshaw et al., 1946; McDermott et al., 1947). Therapeutic successes have been achieved in cases of tuberculous meningitis, miliary tuberculosis and acute, exudative pulmonary tuberculosis. There are certain important therapeutic limitations, however. Tubercle bacilli like other susceptible bacteria tend to develop resistance to streptomycin at a peculiarly rapid rate, both in artificial cultures (Middlebrook and Yegian, 1946) and in the tuberculous host (Youmans et al., 1946). It appears that the development of resistance is due to a selective process—the few naturally resistant individual cells surviving and multiplying in the presence of the drug. Since in the chemotherapy of tuberculosis with streptomycin prolonged and sometimes repeated periods of treatment are necessary, streptomycin-fastness is a conspicuous problem. For the same reason the toxicity of streptomycin for man limits to some extent at present the usefulness of this drug in the therapy of tuberculosis.

Para-aminosalicylic acid, which markedly increases the endogenous respiration of tubercle bacilli, also inhibits their multiplication in vitro and in vivo, appearing to exert

a strong chemotherapeutic effect in mice and in man. This agent has a relatively high degree of specificity in that it inhibits tubercle bacilli in concentrations which have little effect on many other micro-organisms. Its antibacterial activity is said to be antagonized by para-aminobenzoic acid (Lehmann et al., 1946).

#### EPIDEMIOLOGY

Within several species of animals certain experimentally inbred families are consistently more susceptible than others. The genetic factors responsible for these differences have not been determined. However, Lewis and Loomis (1928), and Lurie (1941) have shown that the more resistant families have a greater ability to form antibodies against tubercle bacilli and nonrelated antigens as well as to give a more marked inflammatory response to a wide variety of irritants.

In man, convincing evidence for the role of genetic factors in susceptibility to progressive tuberculous infection has been brought forth by the study of tuberculosis in homozygotic and heterozygotic twins (Kallmann and Reisner, 1943). If one homozygotic twin has clinical tuberculosis, the other twin has 3 chances in 4 of also having clinical tuberculosis, whereas, if one heterozygotic twin has the disease, the other has only 1 chance in 3 of having clinical tuberculosis. Studies aimed at the demonstration of familial differences in susceptibility (Puffer, 1944) are difficult to interpret because of the complicating variable of exposure.

The incidence and type of disease are different in the American Indian, Negro and white races, the former two appearing to be more susceptible (Opie et al., 1936) (Chart 5). These differences are difficult to evaluate because of the differences in living conditions. It is usually assumed that the higher resistance in certain races is the result of a selection pressure occasioned by centuries of endemic infection. It is note-



worthy in this connection that in experimental animals, great differences in susceptibility within the species can be demonstrated by breeding experiments without a natural selective effect of tuberculous infection (Pierce et al., 1947).

Curiously enough there is no striking evidence in experimental tuberculosis that age

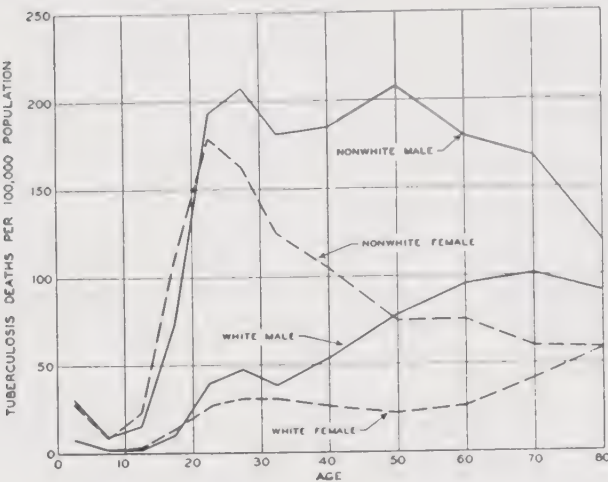


CHART 5. Death rates for tuberculosis (all forms) by sex, race and age in the U. S. (as numbers per 100,000 living individuals in each age group), 1945. (Pitney, E. H., and Kasius, R. V., 1947, Tuberculosis mortality in the United States and in each state: 1945. Pub. Health Rep., 62, 487-511.)

has an influence on the progress of the infection. But, in man it is undoubtedly true that primary tuberculosis in infants has a worse prognosis than in children or adults. The death rate varies with sex and age (Charts 5 and 6). In women the percentage of deaths from all causes reaches the highest level between 15 and 25 years whereas, in males, the rise begins a little later and, as seen in Chart 5, increases more rapidly with age than in females. It is not clear whether the differences between men and women are due to basic physiologic differences associated with sex or to different degrees of exposure and living conditions. Sex differences in susceptibility have not been observed in experimental animals.

It is generally believed that malnutrition

increases susceptibility to tuberculosis. The increase in morbidity and mortality rates during wars in some countries has been attributed to concurrent malnutrition. Overcrowding and other factors were also present and their relative significance is difficult to evaluate. It has not yet been convincingly shown that known vitamins or caloric intake have an influence on tuberculous infection in man or experimental animals. However, some unidentified nutritional factors have recently been found to affect the course of tuberculous infection in mice (Dubos and Pierce, 1948).

The incidence of nonclinical tuberculous infection and of clinical disease are influenced by occupation. Exposure to tubercle bacilli is frequent in the nursing and medical professions. Inhalation of dusts contain-

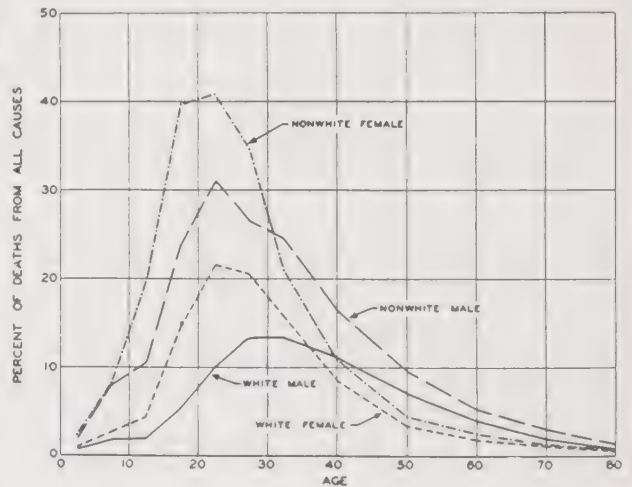


CHART 6. Death from tuberculosis (all forms) as percentages of death from all causes by age, race and sex in the U. S., 1945. (Pitney, E. H., and Kasius, R. V., 1947, Tuberculosis mortality in the United States and in each state: 1945. Pub. Health Rep., 62, 487-511.)

ing silica (silicon dioxide) in certain trades such as granite cutting and certain types of mining, increases the susceptibility to progressive pulmonary disease. This has been and still is an important industrial health problem. In experimental animals the pathologic changes induced by silica accelerate the disease in pulmonary and extrapulmo-

nary sites wherever silica and tubercle bacilli are associated. Silica has no direct effect on the parasite; its local toxic action on the tissue of the host in some way promotes the disease.

The distinction between infection and disease is very significant in the epidemiology of tuberculosis. The infection is widespread in urban populations as indicated by the high percentage of reactors to tuberculin (50 to 95 per cent of adult urban population). Disease, however, at any given time, in the clinically progressive form, is present only in a small percentage of persons infected with tubercle bacilli.

Tubercle bacilli of human type are transmitted primarily by droplet infection from person to person. Bacilli of the bovine type reach man usually by way of unpasteurized milk from the tuberculous cow; they also can be transmitted by the same route as the human type.

Important aspects of the infection essential for the understanding of the epidemiology of the disease are:

(1) Droplet infection originates from persons with draining pulmonary cavities, however small they are. Thus pulmonary tuberculosis, and cavitation specifically, play major roles in the epidemiology of the disease.

(2) Not all infections result in disease recognizable by X-ray or other clinical means. The degree of exposure, however, influences to a great extent the outcome of the infection. Household contact is an important factor in infection.

(3) The disease, particularly in adults, usually has a long and insidious onset.

(4) In some individuals it may be fairly advanced with cavity formation and large numbers of tubercle bacilli in the sputum and yet produce scant, if any, specific clinical symptoms. Like "carriers," these individuals spread the infection.

(5) The progress and type of infection is influenced by many factors such as age and previous exposure. Recovery from one attack of clinical disease modifies susceptibility but does not result in solid immunity.

(6) The reinfection type of tuberculosis commonly seen in adults is in most cases exogenous (see Pathology).

Tuberculosis has been known in urban civilization since the beginning of recorded history. In western civilization the death rate has been declining since 1870, i.e., before the discovery of the parasite (Chart 7).<sup>\*</sup> Nevertheless, in the United States it still has approximately the seventh place among all causes of death, and it is the most frequent cause of death between the ages of 15 and 45 years. It is of great social and eco-

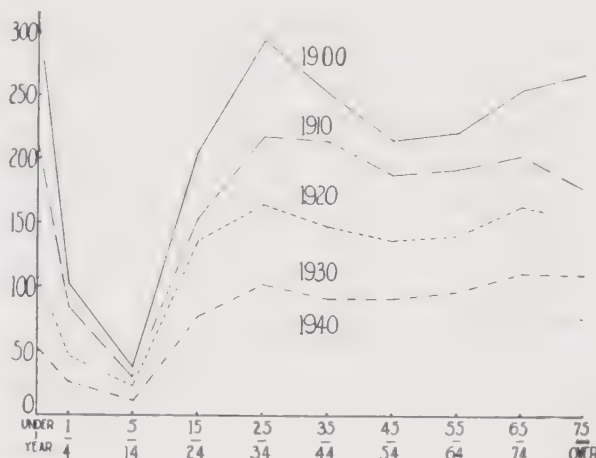


CHART 7. Age-specific death rates of tuberculosis (all forms), U. S. A. in 1900, 1910, 1920, 1930 and 1940. (Vital Statistics, U. S. Department of Commerce, 16, July 16, 1942, No. 7, p. 29.)

nomic significance that many young individuals are incapacitated for long periods of time by tuberculosis, although they may not die of it. The ratio of newly discovered, active cases to deaths per year in the United States is between 7 and 10 to 1. The decrease in mortality rate from tuberculosis in the United States has not been paralleled by an equal decrease in the morbidity rate and prevalence of infection. Therefore, it appears unlikely that the present rate of decline in mortality will continue undiminished. In the Orient, in Central and South America and in Africa the mortality rates are very high and increasing. The prevalence of tuberculosis in various areas is presented in Figure 17.

<sup>\*</sup> The decline in mortality or morbidity rates in western civilization cannot be attributed to a fall in virulence of the parasite.



## CONTROL MEASURES

"The spread of tuberculosis occurs in large part by long drawn-out family or household epidemics in which the disease is slowly transmitted from one generation to the next." (McPhedran and Opie, 1935.) This observation serves as a basis for most

school and college students), applicants at larger organizations, governmental or private, are becoming recognized routine measures. For case finding, the tuberculin test and X-ray examination are used. The value of the former consists in reducing the necessary numbers of X-ray examinations in groups with a low rate of tuberculin posi-



FIG. 17. Prevalence of tuberculosis as estimated from the probable death rates from all forms of tuberculosis in the various countries of the world. (Yelton, S. E., 1946, *Tuberculosis throughout the world*. Pub. Health Rep., 61, 1144-1160.)

of the measures used in public health control. Early diagnosis gives not only the best chance for effective treatment but also the best opportunity to prevent the spread of the disease to possible contacts. For diagnosis of the disease which is often advanced, yet asymptomatic, special public health measures are required. It is necessary to examine persons whose risk of contracting the disease is relatively high, such as household and other contacts of known cases of tuberculosis (nurses, medical students, house officers of hospitals). Periodic examinations of adolescents and young adults (high

tive persons since these do not require X-ray examinations.\* Positive X-ray findings have to be supplemented by clinical and laboratory examinations.

The need for case finding work is clearly brought out by the fact that more than half of the cases diagnosed in ordinary medical practice are moderately or far advanced at the time of diagnosis. During World War II, in Massachusetts, 1.4 per cent of the persons examined at induction centers for

\* The percentage of reactors to tuberculin indicates the prevalence of infection in the group—in formation of great epidemiologic interest

the armed forces had lesions demonstrable by X-ray (Zacks et al., 1944). It may be noted here that in about one half of the cases discovered by X-ray the infection does not progress and treatment is not required; unfortunately, it is not possible to predict which individuals are in this group. Segregation and treatment of incipient cases is best done in sanatoria, the hospitals being used, as a rule, for the treatment of advanced cases. It is in sanatoria that the patient can be educated not only to take care of himself but also to prevent the spread of tubercle bacilli to others. Unfortunately, a large number of patients cannot be admitted to sanatoria or hospitals or have to be discharged from such institutions before they are cured because of the shortage of facilities. It is estimated that in the United States there is a need of 50,000 additional beds to take care of the tuberculous patients. Once the diagnosis of tuberculosis is made, it is essential to examine periodically household and, often, other contacts. If the patient remains at home, the family has to be educated as to the nature and spread of the disease, and very often as to personal hygiene and disinfection of objects contaminated with tubercle bacilli. Economic problems are often conspicuous, particularly when either of the parents is diseased.

In the prevention of the disease vaccination may come to play an important part in the future (see Immunization).

For the planning of control measures against this reportable disease accurate statistics on morbidity and mortality with reference to age, sex and occupation are necessary. Such information is collected by city, county, state, Federal governmental agencies and life insurance companies. Control measures are carried out by governmental and voluntary agencies. The Federal government gives financial aid to the state departments of health. The funds are allocated to the states by the U. S. Public Health Service, Division of Tuberculosis. This division is engaged in various activities such as statistical studies, laboratory research, demonstration of the value of case finding, promoting studies on vaccination and

subsidizing research on tuberculosis. The state, county and city departments of health are engaged in maintaining tuberculosis hospitals and clinics with particular emphasis on case finding among the contacts, offering diagnostic, consulting service to private practitioners. The public health nurses visit families to arrange for hospitalization, to obtain economic aid for the families from governmental or private welfare organizations; they educate the families with reference to the care of patients, isolation technic, and personal hygiene. The public health nurses are particularly helpful in the follow-up of patients and contacts.

The activities of voluntary private agencies are manifold. The largest private organization is the National Tuberculosis Association, which is supplemented by local associations. They carry out much educational work by arranging for public speakers, moving pictures and distributing literature on the subject of tuberculosis. The voluntary organizations are active in vocational guidance and rehabilitation. The National Tuberculosis Association gives financial and other aid for research and postgraduate training of physicians.

The reduction of prevalence of tuberculosis caused by the bovine type during the past decades is obviously due to measures taken against tuberculosis in dairy herds and to pasteurization of milk. The control measures (early diagnosis, segregation, etc.) directed against tuberculosis caused by the human type could not have failed to contribute a great deal to the phenomenal reduction in its prevalence during the past 50 years. It is likely, however, that other factors had an even greater effect. The frequency of this disease in different groups of people varies with their economic status. It is probable that the great rise in the standard of living (less crowding in the households, better nutrition, better education as to personal hygiene, etc.) during this century had a large share in diminishing the prevalence of the disease. It is difficult to establish the comparative values of the specific public health measures and the rise in standard of living in combating tuberculosis.



## TUBERCULIN

Tuberculin is a mixture of protein fractions of tubercle bacilli.

"Old tuberculin" (O.T.) is prepared in the following way. Bacterial cells are grown on a glycerin-broth medium for about six weeks; the cultures are evaporated at 100° C. to one tenth of the original volume and passed through a filter to remove the bacteria. The order of heating and filtering may be reversed. It is possible to obtain tuberculin in relatively pure form by growing tubercle bacilli on synthetic media and precipitating the protein fractions. These fractions are heated to decrease their capacity to sensitize. Such a preparation known as Purified Protein Derivative (P.P.D.) (Seibert et al., 1934) is becoming widely used. Both the "old tuberculin" and P.P.D. have to be standardized in guinea pigs or man since biological activity varies with different lots.

Tuberculins made with human or bovine strains cannot be readily distinguished from each other but are quite different from avian tuberculins.

For the skin test one tenth of a milliliter of a high dilution of tuberculin is injected into the skin over the forearm. (Mantoux or intradermal test). It is customary to use first a small dose, 0.01 mg. (1:10,000 dilution), and if there is no reaction, a larger dose, namely 1 mg. (1:100 dilution). The P.P.D. preparation is used in amounts which produce reactions corresponding in intensity to 0.01 and 1 mg. O.T. The tuberculin reaction is characterized by delayed appearance and relatively long duration. It usually appears several hours after injection and the maximum response may be seen in one or two days. The reaction is positive if there is an induration more than 10 mm. in diameter; erythema is usually present but is not considered in judging the size of the reaction which is graded according to the longest diameter of the area indurated. A papule may develop in the central part of the indurated area and necrosis may occur here. Weak reactions may appear and disappear faster than the stronger reactions.

No control is necessary for the intracutaneous tuberculin test.

Other forms of skin testing have been suggested to avoid the use of injections. The oldest is the von Pirquet scratch test which consists of rubbing tuberculin into a scratched area; tuberculin may be incorporated into an ointment and used as a patch test. These methods of skin testing are less quantitative and less sensitive than the intracutaneous test.

A positive tuberculin test indicates that the reactor has been infected but does not necessarily indicate disease; it may be positive in the absence of lesions recognizable by X-ray. Lack of reaction indicates absence of infection with the following qualification: the person may be in the preallergic state during the early stage of first infection (a period not exceeding a month), or may have lost allergy due to overwhelming infection. The latter is uncommon. In general, persons with active lesions and those exposed recently and often (household contacts) react to smaller doses of tuberculin than others (Furcolow et al., 1941). However, intensity of a positive reaction in individual cases is of little significance. The tuberculin test is of great value in excluding tuberculosis, and also in epidemiologic studies to indicate the prevalence of infection. Loss of tuberculin sensitivity in persons with healed tuberculosis of first infection has been observed repeatedly in recent years. It is possible that casual contacts with tubercle bacilli are becoming less frequent and thus one factor in conditioning the maintenance of tuberculin allergy is becoming less prevalent. Tuberculin sensitivity probably is lost in persons who develop Boeck's sarcoid (Reisner, 1944).

When a large amount of tuberculin, 0.5 of a milliliter of undiluted O.T. for instance, is injected into a tuberculous guinea pig a systemic reaction ensues quite gradually: the tubercles and surrounding tissues become congested and often hemorrhagic, exudate appears in the peritoneal and pleural

cavities, and the animal dies in one or two days. Anaphylactic symptoms fail to appear. Systemic tuberculin reactions may be produced in hypersensitive human beings, and untoward effects may be produced with excessive doses in ocular tuberculosis and tuberculous cervical adenitis.

#### BACTERIOLOGIC DIAGNOSIS

As already discussed, hypersensitivity of the skin to tuberculin indicates infection, but not necessarily disease. A negative test usually rules out infection. Positive tuberculin reactions in infants suggest progressive disease because of their high susceptibility. Conversion from negative to positive signifies recent exposure and infection; frequent examinations of such cases are indicated.

Bacteriologic diagnosis rests on the demonstration of virulent tubercle bacilli. Direct smear revealing acid-fast rods is usually reliable especially when present in sputum or spinal fluid. In view of the fact that very large numbers of bacilli are necessary before they can be detected in the usual search of a direct smear, cultivation technics often have to be resorted to. Animal inoculation may be necessary to establish beyond question that an acid-fast rod is a tubercle bacillus.

**Direct Smear.** Morning sputum or a collection of sputa over a period of days is examined first macroscopically for possible presence of particles which should be selected for examination. In the absence of such particles the sputum may be directly smeared and examined.

**Concentration and Smear.** If the direct smear is negative, the sputum is digested by some agent such as Chlorox (hypochlorite solution) which liquefies the mucoid material and allows centrifugalization of particulate matter. This sediment can be smeared.

**Culture.** If the results of the above procedures are negative, or if it is desired to prove that an acid-fast rod is a tubercle bacillus (usually unnecessary), the sputum can be liquefied by any one of various agents (so-

dium hydroxide, sulfuric acid, oxalic acid, trisodium phosphate) which are bactericidal for many contaminating micro-organisms but less so for tubercle bacilli. The liquefied sputum is centrifugalized and the sediment planted on appropriate media. The cultures should be incubated and examined at intervals for a few weeks. Nonpathogenic mycobacteria should not be confused with pathogenic strains (see Cultivation).

**Animal Inoculation.** In some laboratories a portion of the material prepared for culture is inoculated into the groin of young guinea pigs. The animals are tested with tuberculin at intervals of a few weeks and examined for tuberculosis when the reaction becomes positive. The relative value of the cultural and the animal inoculation methods vary in different laboratories. The cultural method is more exclusively used.

The general technics described above for sputum examination are also applicable to the examination of gastric contents (particularly in infants and when the sputum is negative or when it is not obtainable), pleural fluid, urine (smegma bacilli may frequently be found in urine sediments), and spinal fluid. The fibrin web which forms after brief incubation of spinal fluid may be directly transferred to a slide and examined, cultured and inoculated into guinea pigs. Biopsy material may be macerated and subjected to the above procedures.

Positive findings are proportional to the frequency of examinations. One or two negative findings do not rule out tuberculosis.

#### TUBERCULOSIS IN ANIMALS

The host range of tubercle bacilli is almost unlimited. The mammalian types can infect domestic and wild animals in captivity. Avian tubercle bacilli infect, besides fowl, swine and, rarely, cattle, sheep and horses. In aquaria occasionally fishes and turtles may be found to have progressive tuberculosis caused by mycobacteria pathogenic for poikilothermic animals.

A large number of cattle are infected with the bovine type of tubercle bacilli and many have progressive disease. Tubercle bacilli are often present in the milk of infected cows even if tubercles are not demonstrable



in the udder.\* Most infected cattle in the United States have no gross pulmonary tuberculosis but only infected lymph nodes; generalized tuberculosis is not frequent. The infection is diagnosed by the subcutaneous tuberculin test (local inflammatory reaction often associated with fever). The demonstration of tubercle bacilli in the milk may be used for the diagnosis of the disease. For the eradication of cattle tuberculosis in the United States, herds are tuberculin tested periodically and the reactors are slaughtered. The rate of infection in the United States was reduced from about 4 per cent in 1917 to about 0.5 per cent in 1940. In other countries less expensive methods are used such as slaughtering of grossly tuberculous cows and segregating the reactors.

Tuberculosis in the chicken is most conspicuous in the liver, but the spleen, intestines, lungs and other organs may be affected. Avian tubercle bacilli may be present in hens' eggs. The infection may be diagnosed by injection of avian tuberculin into the wattle. According to a survey in 1930 in the United States, about 5.8 per cent of chickens were infected with tuberculosis (more than 8 million fowl). Avian tubercle bacilli are not pathogenic for man (see Types of Tubercle Bacilli).

Pigs are infected usually with bovine or avian tubercle bacilli and rarely with the human type. Infection results from ingestion of contaminated milk or material contaminated with feces of tuberculous fowl. In most pigs the infection is localized to the lymph nodes of the alimentary canal.

### JOHNE'S DISEASE

This is a specific enteritis of cattle, sheep and deer caused by an acid-fast bacillus called Johne's bacillus (*M. paratuberculosis*). The disease has a long incubation period, runs a very chronic course charac-

terized by intermittent diarrhea and progressive emaciation without fever. In certain countries it causes a great economic loss in cattle.

*M. paratuberculosis* is a short thick rod, which is acid fast and does not form spores. On primary isolation it can be cultivated only on media containing an as yet unidentified substance or substances present in acid-fast bacilli or in alcoholic extracts of certain plants (Twort et al., 1912). The vitamin K type of substances have a growth-promoting effect (Wooley and McCarter, 1940). The lesions are characterized by gross thickening of the mucosa of the small intestine and enlargement of the mesenteric lymph nodes without ulceration. The cellular reaction about the bacilli which may be intracellular or extracellular, is diffuse, not localized as in tuberculosis. Lymphoid and epithelioid cells are present; giant cells are rare; caseation and calcification do not occur.

### LEPROSY

Since it is characteristically present in leprosy lesions, *M. leprae* (Hansen's bacillus), has been accepted as the etiologic agent of leprosy, although cultures from leprosy lesions or the lesions themselves introduced into experimental animals do not produce the disease. Several strains of acid-fast organisms have been cultivated from leprosy lesions, but those which appear to be especially significant are difficult to propagate in successive cultures. Sera of patients with leprosy do not react with the so-called *M. leprae* cultures.

In histologic sections, leprosy bacilli are from 1.5 to 8  $\mu$  long and from 0.2 to 0.5  $\mu$  in diameter. They are straight or slightly curved and often occur in globular masses, known as globi, and in groups arranged as parallel rods. As a rule, leprosy bacilli are stained uniformly red with carbol fuchsin, but occasionally granules can be seen.

The disease occurs in nodular (cutaneous) and neural (anesthetic) forms: the two

\* In the milk of tuberculous women tubercle bacilli are very rarely found unless there are tubercles in the mammary glands, and this is very uncommon.

forms are often present in the same patient. In nodular leprosy the skin is raised over firm nodules (lepromata) which are most frequently present on exposed parts such as the face and extremities. The skin often ulcerates and secondary infections may set in. The neural form affects peripheral, particularly sensory, nerves producing anaesthesia. Due to lack of sensation, the affected hands and feet are likely to be injured leading to mutilation of these parts. Lesions may develop in almost all organs except voluntary muscle. The cartilage of the nose is often destroyed, the mucous membranes of the mouth and nose, the eye and the testes are frequently affected. Lesions are common in lymph nodes. They are characterized by granulation tissue well supplied with blood vessels and lymph ducts. There are many large mononuclear cells containing numerous acid-fast bacilli and fat globules. Multinucleated giant cells are often present. Most of the acid-fast bacilli are in the endothelial cells of the lymph ducts and blood vessels. In the neural lesions, the bacilli are in the perineural and epineural tissue producing extensive formation of granulation tissue. The nerve fibers degenerate with loss of motor function as well as sensation. The hands and feet may, therefore, undergo atrophy.

The disease usually progresses very slowly; the neural type is particularly insidious. Remissions oftentimes occur; this fact has made evaluation of possible therapeutic measures very difficult. Chaulmoogra oil and its derivatives have been used for many years in the treatment of leprosy, but not until the advent of the sulfonamidelike compounds and streptomycin have unequivocal chemotherapeutic effects been demonstrable. The sulfones and streptomycin are now used in the treatment of certain forms of leprosy (Faget and Erickson, 1948).

The bacteriologic diagnosis is most often made by finding acid-fast bacilli in scrapings of the nasal mucosa and in the tissue fluid expressed after superficial incisions of

the skin in certain areas. There is no serologic test of value. Extract of leprosy tissue containing *M. leprae* has been employed as a skin test, but the value of the reaction to "lepromin" or "nastin" has not been well defined. It may be noted that a large percentage of leprosy patients who have neither syphilis nor yaws are Wassermann positive.

The disease is believed to have originated in Central Africa; from there it spread to all parts of Europe and subsequently to the United States. It is widely disseminated in the Orient. Leprosy was introduced to the Hawaiian Islands during the nineteenth century. It is estimated that there are 3 million lepers in the world, about 750 in the United States and over 30,000 in Central and South America. It is interesting that, in contrast to tuberculosis, this disease is widespread in nonurban civilization.

All human races are susceptible to the disease, and it is said that susceptibility is genetically influenced. This opinion is based on the greater incidence of the disease in siblings than in husbands and wives within the same family. The susceptibility, however, is greatest in childhood; thus, this conclusion may not be a valid one. The ratio between the incidence of the disease in males and in females is approximately 2 to 1.

The incubation period varies from a few months to many years, most commonly from two to four years. The ulcers of the skin and the nasal discharge are probably the most important sources of infection. The disease is not highly contagious and is probably acquired by infecting superficial abrasions of the skin. It is likely that persons can acquire the infection without developing manifest disease. It has not been shown that animal vectors spread the infection. The most effective prevention is segregation of patients in leprosaria. The removal of children from leprosy parents has proved to be a useful prophylactic measure.

Unlike the tubercle bacillus, *M. leprae* of



man does not produce progressive infection in animals. But rats in many countries have a disease called "rat leprosy," which is characterized by nodular skin lesions containing acid-fast bacilli which are difficult to cultivate and are nonpathogenic for other species of animals. There is no evidence indicating that rat leprosy can be transmitted to man or that the rat plays any role as a vector for human leprosy.

It is interesting to compare *M. tuberculosis* and *M. leprae*. Tubercle bacilli have many types, and their host range is almost

unlimited; they are easy to cultivate on artificial media. There are only two types of *M. leprae*; they are exclusively pathogenic either for man or the rat; and cultivation of *M. leprae* is difficult or impossible. Tuberculosis has been the disease of urban civilization and occurs in domestic animals. Leprosy is prevalent in the tropics among people who live under rural conditions. After its introduction to Europe from the Near East, the disease at first became endemic but later became almost extinct (Strong, 1942, chapter on leprosy).

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# 13

## The Staphylococci

### INTRODUCTION

The staphylococci are Gram-positive spherical cells typically occurring in irregular clusters. They are facultatively anaerobic, although rare species are strictly anaerobic. They grow well on artificial media. Their biochemical and biologic properties are variable, but they commonly ferment various carbohydrates, liquefy gelatin and hemolyze blood. Growth on solid media varies in color from deep gold to white, less often lemon yellow. Staphylococci are normally parasitic on skin and mucous membranes and responsible for many common suppurative infections. The strains pathogenic for man and animals are coagulase-positive. Representative of the pathogenic staphylococci is *Micrococcus pyogenes* var. *aureus* (Rosenbach) Zopf.

The staphylococci are members of a large group of "micrococci," many species of which are saprophytes and are similar morphologically to the staphylococci. Until recently the staphylococci were classified as a separate genus of the family *Micrococcaceae*, the representative species being *Staphylococcus aureus*. They have now been assigned to the genus *Micrococcus* in the same family, the principal pathogenic species being designated as *Micrococcus pyogenes* var. *aureus* (Bergey's Manual, 1948). The custom of referring to the pathogenic micrococci as "staphylococci," supported by constant use since the term was first employed some sixty years ago, will undoubtedly persist, and the term "staphylococcus" will be used in the following discussion.

Infections produced by the staphylococci present a variety of clinical and pathologic forms, often characterized by suppuration and ranging from mild localized pustules to ful-

minating, rapidly fatal septicemia. Furuncles and carbuncles, osteomyelitis and many abscesses of soft tissues are due to them; they are frequently responsible for purulent infections of traumatic and surgical wounds, and occasionally for pneumonia, meningitis or suppurative infections of the pleural, peritoneal and synovial cavities. The most common type of bacterial food poisoning is caused by staphylococci.

### HISTORY

Micrococci which were probably staphylococci were seen in pus and were cultivated by several of the early bacteriologists. Because of the resemblance of their clusters to a bunch of grapes, Ogston in 1880 devised the name "staphylococcus" from the Greek word for grape, "staphulé." The staphylococci were isolated and systematically studied in 1884 by Rosenbach, who identified the *aureus* and *albus* forms; other varieties were studied and named in the ensuing years. By the turn of the century, it was recognized that staphylococci were capable of forming toxic substances, but these toxins received surprisingly little attention until about twenty years ago. The demonstration by the Dicks that streptococci from scarlet fever produce an erythrogenic toxin stimulated Parker (1924) to reinvestigate the formation of exotoxin by staphylococci. Burnet (1929) was led to a similar study by the occurrence in 1928 in Bundaberg, Australia, of a group of fatal systemic



staphylococcal infections resulting from the prophylactic injection of a diphtheria toxin-antitoxin mixture which had become contaminated by staphylococci. The therapeutic use of staphylococcal toxoid and antitoxin may be traced to Burnet's fundamental studies. Following the work of Dack and others beginning in 1930, staphylococcal enterotoxin was differentiated from other toxic factors, and food poisoning due to the staphylococci became recognized as a clinical entity. The staphylococci played a role in the discovery of penicillin, for it was on a plate culture of staphylococci contaminated by *Penicillium notatum* that Fleming in 1929 observed the antibacterial effect of the mold.

### DISTRIBUTION

The micrococci are widely distributed and are constantly present in man's environment, being found in the air, dust, water, on articles of daily use and on the skin. These environmental forms are usually saprophytic. The parasitic staphylococci form a part of the permanent bacterial flora of the skin and the nasopharynx. On the skin they frequently lodge in hair follicles and ducts of sebaceous glands. Potentially pathogenic forms are constantly carried on the skin or in the nose by approximately 20 and 50 per cent, respectively, of all individuals. When pathogenic staphylococci are

found in the environment they may be assumed to have been derived from a human or animal source.

Spontaneous staphylococcal infections occur in both man and animals. Although man is especially susceptible to them, it is not uncommon to encounter staphylococcal abscesses of the soft tissues or bone among cattle, horses and other domestic animals. While bovine mastitis is primarily a streptococcal disease, an appreciable number of cases are caused by staphylococci.

### MORPHOLOGY

Typically the staphylococci are spherical cells which are grouped in irregular clusters. In preparations made from broth cultures the clusters are small, and single cocci, pairs or short chains may be seen; long chains are never found. Stained preparations from agar characteristically show larger clusters of cocci. In direct-stained preparations of pus or other pathologic material the grouping is similar to that seen in fluid media. The average size of the cocci is about 0.8 to 1 micron. On the whole, the staphylococci in any given culture tend to be smaller and more uniform in size and arrangement than the purely saprophytic micrococci. They do not form spores, are not motile, and stain readily with the usual basic anilin dyes, less easily with some acid dyes. The cocci in



FIG. 18. Morphology and grouping of staphylococci. (Left) staphylococci in pus; (center) 18-hour culture in broth; (right) 18-hour culture on agar. Gram-stained preparations.

cultures 24 hours old or less are invariably Gram positive; individual cocci in older cultures occasionally exhibit some variability in their Gram staining.

### CULTIVATION

The staphylococci grow abundantly on the usual meat extract and infusion media. Both thiamine and nicotinic acid are essential growth factors, while uracil is necessary for anaerobic growth. The optimum temperature for growth is 37° C., but slow development occurs at 10° C. and 42° C. Growth takes place nearly as well at moderately alkaline and acid reactions with an optimum at pH 7.4. The staphylococci are preferably aerobic but grow also in the absence of oxygen; an atmosphere containing a mixture of air and 30 per cent carbon dioxide is particularly favorable for the production of staphylococcal toxin. A few species of staphylococci are strictly anaerobic, but are not commonly encountered.

On agar the colonies are round, opaque, raised, smooth and glistening, and exhibit characteristic pigmentation which ranges from golden or yellow to white. The following species are recognized: *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*)—golden; *Micrococcus pyogenes* var. *albus* (*Staphylococcus albus*)—porcelain white; *Micrococcus citreus* (*Staphylococcus citreus*)—lemon yellow; *Micrococcus aurantiacus* (*Staphylococcus aurantiacus*)—yellow gold; and *Micrococcus epidermidis* (*Staphylococcus epidermidis*)—white. The pigment is best developed on agar containing a carbohydrate. The shade of pigment produced by the *aureus* variety ranges from deep gold to pale cream. While the color is usually distinct after incubation for 24 hours at 37° C., it is intensified when the culture is held at room temperature for another day or two. No pigment is produced anaerobically or in broth. The growth in broth is uniformly turbid with an amor-

phous, occasionally stringy, sediment. The majority of pathogenic staphylococci are of the *aureus* variety, less often *albus* and rarely *citreus*; the other two species sometimes are responsible for relatively mild infections.

### BIOCHEMICAL REACTIONS

In general, gelatin is liquefied; carbohydrates, particularly glucose, lactose, sucrose and mannitol, are fermented with the production of lactic acid but no gas; nitrates are reduced to nitrites; milk is acidified and sometimes coagulated; and indole is never formed. The metabolic capacities of staphylococci vary considerably from one strain to another, a fact which reduces the significance of these reactions as a means of identification. However, it should be pointed out that the pathogenic staphylococci tend to exhibit a fuller complement of biochemical reactions and to show greater metabolic activity than the nonpathogenic varieties; the pathogens are usually more deeply pigmented, liquefy gelatin more rapidly and a larger proportion of them ferment mannitol. Blood agar is often hemolyzed by staphylococci, this reaction being subject to the same variations as are the biochemical properties.

### RESISTANCE

The staphylococci are relatively more resistant than many of the other non-spore-forming bacteria and remain viable for many weeks when dried in pus, on cloth or other vehicles. Agar cultures are easily maintained for several months at room or refrigerator temperatures. The thermal death point is in the neighborhood of 60° C., exposure to this temperature for one-half to one hour often being necessary to kill them. Certain dyes, particularly gentian violet, are strongly bacteriostatic for staphylococci even in low concentrations. The staphylococci are far less sus-



ceptible to the bacteriostatic action of the sulfonamide drugs than the beta-hemolytic streptococci, pneumococci or gonococci. Many are inhibited by penicillin, tyrothricin and streptomycin. Depending on the concentrations employed, the action of the antibiotics is either bacteriostatic or bactericidal. Staphylococci killed by penicillin undergo lysis. Strains which are naturally resistant to the antibiotics are not uncommon, while resistance is frequently acquired upon exposure to antibiotics either in vitro or in vivo. The acquisition of resistance to penicillin appears to result from the progressive selection of the more resistant forms which are normally found in any given culture. Some of the strains which are naturally resistant or which acquire resistance in vivo, i.e., during penicillin therapy, produce a penicillin-inhibitor, penicillinase.

#### ANTIGENIC RELATIONSHIPS

Staphylococci produce cellular polysaccharides which are distinguished by the end products of their hydrolysis, by their optical rotation and by specific precipitation with immune sera. It is possible to separate the staphylococci by means of the precipitation reaction with these polysaccharides into two large groups which have been designated as Type A (pathogenic) and Type B (nonpathogenic) (Julianelle and Wiegand, 1935). The polysaccharides are not themselves antigenic, the immune sera being obtained by immunization of rabbits with whole cultures of the cocci. Both Types A and B staphylococci contain a protein which is antigenic but which, being common to both, does not serve to distinguish between the types. Thompson and Khorazo (1937) and Cowan (1938) found evidence for the existence of additional groups which were distinguished from Types A and B by precipitation reactions. Cowan's additional group represents a further division of the pathogenic staphylococci, while that of

Thompson consists essentially of nonpathogens. Since both were designated as Type C, some confusion is likely to result until their position is more exactly defined. Specific proteins for Types A, B and Thompson's Type C were described by Verwey.

A rough, although not entirely satisfactory, distinction between pathogenic and nonpathogenic strains is obtained by simple agglutination, for immune sera which agglutinate pathogenic staphylococci do not as a rule agglutinate the nonpathogenic varieties. Early attempts to use the technique of agglutinin absorption suggested not only a broad distinction between pathogens and nonpathogens but also subdivisions within these groups. Using agglutinin absorption and slide agglutination, Cowan (1939a) and Christie and Keogh (1940) established the existence of nine serologic subgroups among the pathogenic staphylococci and it is likely that more will be found. The serologic identification of subgroups of staphylococci promises to be of chief value in epidemiologic studies; a few such studies have been reported.

Recently, identification of staphylococci has been attempted by bacteriophage "typing" in a manner similar to that used by Craigie with the typhoid bacillus. The method involves the determination of similarities or differences in the susceptibility of cultures of staphylococci to a series of staphylococcal bacteriophages. By this means the identity of cocci from different sources or of strains isolated from an individual at different times has been demonstrated (Fisk and Mordvin, 1944). Bacteriophage typing promises to be useful in studying the epidemiology of staphylococcal infections; a few suggestive studies on staphylococcal food poisoning, osteomyelitis and other infections have been reported.

#### DISSOCIATION

The dissociation of staphylococci sometimes occurs spontaneously in cultures and

has been produced experimentally by exposure of the cocci to suitable concentrations of barium or lithium chloride, to bacteriophage, gramicidin or penicillin. The variants are distinguished by alterations in the pigmentation of colonies, such as a change from golden to white in the case of *aureus* strains, or by the development of rough, mucoid or minute translucent colonies from normally smooth cultures. The splitting off of *albus*, *citreus* and *roseus* colonies from a normally golden strain has been described. In addition to changes of color, variants exhibit a reduction in their capacity to ferment carbohydrates, liquefy gelatin or hemolyze blood. Pathogenicity for experimental animals and the ability to produce exotoxin may be lost; however, some of Burnet's best toxin-producers were *albus* variants of *aureus* strains. Antigenic differences between the parent strain and the variants are shown by agglutination and agglutinin absorption tests. Differences in the chemical composition of variants and the parent strain are suggested by the work of Hoffstadt and Clark (1938). Small-colony variants obtained by exposure to antibiotics may exhibit a nonspecific drugfastness; those produced by exposure to penicillin, for example, show an enhanced resistance to both penicillin and methyl violet.

### PATHOGENESIS

The staphylococci elaborate several toxins and enzymes which contribute in varying degrees to their ability to produce infection (Blair, 1939). A soluble, filterable, thermolabile exotoxin is produced which causes tissue necrosis or death in experimental animals and hemolyzes rabbit erythrocytes. Exotoxin is antigenic, giving rise to a specific antitoxin which neutralizes it according to the law of multiple proportions; the potency of antitoxin is measured in terms of a standard international unit. An antigenic toxoid is prepared by treating staphylococcal exotoxin with formalin.

Three distinct hemolysins (hemotoxins) have been designated as  $\alpha$ -hemolysin,  $\beta$ -hemolysin and  $\gamma$ -hemolysin (Glenny and Stevens, 1935; Llewellyn Smith and Price, 1938). Rabbit erythrocytes are particularly susceptible to lysis by  $\alpha$ -hemolysin; sheep cells are sometimes hemolyzed, but human erythrocytes are relatively resistant to its action. Lysis of rabbit cells is produced in one hour at 37° C. by culture filtrates which contain the lethal, necrotic toxin. Since the three factors are usually found in approximately constant quantitative relationships in toxic filtrates and are quantitatively neutralized by antitoxin, it is generally assumed that exotoxin is a single entity which is capable of producing all three reactions: hemolysis of rabbit cells, tissue necrosis and death; however, some evidence to the contrary has been reported. Exotoxin which hemolyzes rabbit erythrocytes is produced chiefly by staphylococci which are pathogenic for man. The  $\beta$ -hemolysin produces lysis of sheep cells upon incubation for one hour at 37° C., followed by incubation for 18 hours at 10° to 22° C., so-called "hot-cold" lysis. Bovine erythrocytes are as susceptible to  $\beta$ -hemolysin as are sheep cells, while rabbit and human cells are less readily hemolyzed. The  $\beta$ -hemolysin is produced chiefly by staphylococci of animal origin and only occasionally by human strains; it is unrelated to the pathogenicity of staphylococci for man. The  $\gamma$ -hemolysin also hemolyzes rabbit cells, but it appears to be distinct from  $\alpha$ -hemolysin and has not been shown to be related to the pathogenicity of staphylococci for man. The three hemolysins are antigenically distinct.

The first toxic substance of staphylococci to be identified and studied, leukocidin contributes to their pathogenicity because of its destructive action on the leukocytes (Van de Velde, 1894). Leukocidin is produced in varying amounts by the majority of pathogenic staphylococci, particularly by those which are actively capable of invading the tissues. It is formed independent of exo-



toxin. Both leukocidin and exotoxin are often present in the same culture filtrate, and both destroy rabbit leukocytes; because of this, the effect on leukocytes as determined by the Neisser-Wechsberg technic, which is based upon the capacity of living rabbit leukocytes to reduce methylene blue, has led to the assumption that the two factors are identical. That leukocidin is distinct from exotoxin is shown when the method of Valentine (1936) is used, involving the direct microscopic examination of stained preparations made from a mixture of culture filtrate and human blood. Since human leukocytes are affected by leukocidin but not by exotoxin, any destruction of the leukocytes which is observed may be attributed to the action of leukocidin. Leukocidin is soluble, filterable, more labile than exotoxin, and is antigenic.

Certain strains of staphylococci produce an enterotoxin which is responsible for the acute gastro-intestinal symptoms of food poisoning (Dack, 1943). Although enterotoxigenic strains are widely distributed, they appear to comprise a relatively small proportion of all the pathogenic staphylococci. By coincidence, many food poisoning strains produce exotoxin as well as enterotoxin. The enterotoxin, however, is a distinct entity and is distinguished from other staphylococcal toxins by certain physical properties and by the fact that it is not neutralized by antisera which neutralize exotoxin. A characteristic property of enterotoxin is its thermostability; it resists boiling for 30 minutes, in contrast to the ready destruction of exotoxin at about 60° C. and a low degree of heat-stability of  $\beta$ -hemolysin.

A characteristic and essentially an exclusive property of the staphylococci is their ability to clot blood plasma. Clotting is produced by an enzyme, coagulase, which is formed only by pathogenic staphylococci of either human or animal origin; nonpathogenic strains are unable to clot plasma. Because of this, it is possible to distinguish

between pathogens and nonpathogens by a simple laboratory test for clotting; the reaction is recognized as a reliable *in vitro* criterion of the potential pathogenicity of staphylococci (Cruikshank, 1937). Details of the test are given below; briefly, it is based on the fact that when a mixture of culture and plasma is incubated at 37° C. a clot is formed by pathogenic staphylococci in the plasma in three hours or less. Rabbit or human plasma is readily clotted, other animal plasmas varying in their susceptibility to the action of coagulase. Under suitable conditions, solutions of fibrinogen may be clotted. It has been suggested that coagulase itself is inert so far as the actual conversion of fibrinogen into fibrin is concerned, and that it is the precursor of a thrombinlike substance which requires the participation of an activator (Smith and Hale, 1944). The activator, the nature of which is unknown, is present in adequate amounts in some plasmas, e.g., rabbit or human, but is lacking or inadequate in others. The presence or absence of activator would explain the susceptibility or resistance of various animal plasmas to clotting and the individual variations which are observed among plasmas from one species. Coagulase is filterable and thermostable; it does not appear to be antigenic.

Fibrin clots are dissolved by an enzyme, fibrinolysin (Fisher, 1936). Among the staphylococci the ability to cause fibrinolysis is essentially confined to pathogenic (coagulase-positive) strains of human origin; only rare strains from animal sources dissolve fibrin clot. In contrast to the rapid fibrinolysis produced by  $\beta$ -hemolytic streptococci, often a matter of only a few minutes, lysis of clots by staphylococci requires from several hours to two or three days. When the clot formed in plasma by a coagulase-positive strain is incubated for an extended period, lysis is sometimes, but not consistently, obtained. Lysis is best demonstrated by adding staphylococci to plasma or fibrinogen solution, which is then imme-

diately clotted by the addition of calcium or thrombin and incubating the mixture at 37° C. Clots of human and rabbit fibrin are particularly susceptible to lysis by staphylococci, other animal fibrins being wholly or variably resistant. Fibrinolysin is filterable, thermostable and antigenic.

In common with some other pathogenic bacteria, staphylococci produce a spreading factor (Duran-Reynals, 1942) which, by increasing the permeability of connective tissue, aids in the initial formation of the local lesion. The spreading factor is related to the mucolytic enzymes, or hyaluronidases, and is similar to a spreading factor found in testicle and other body tissues. The action of the spreading factor is demonstrated in vivo by the intradermal injection in a rabbit of a mixture of India ink and an extract of staphylococci. In the presence of spreading factor the ink diffuses rapidly from the site of injection. Spreading factor is soluble, filterable and appears to be relatively heat stable; its antigenicity is at present in doubt. The spreading factor is not itself toxic but facilitates the dispersion of the cocci and their toxic products. The extent and severity of the local lesion are related to the ability of the cocci to elaborate spreading factor. Experimental infections with other bacteria or with viruses are enhanced by staphylococcal spreading factor.

The work of Lyons (1937) suggests that possession of a capsule by staphylococci enhances their pathogenic capacity by rendering them resistant to phagocytosis. Lyons reported that capsules were demonstrable in young (3-hour) cultures but were not found in older forms. In defibrinated blood, a serum component prevented the formation of capsules, and nonencapsulated staphylococci were readily ingested by leukocytes. Within the leukocytes, where they were protected from the serum, the cocci reproduced their capsules and strains which formed leukocidin destroyed the leukocytes. When encapsulated staphylococci were sensitized

by a specific anticapsular antibody they were readily ingested and destroyed by leukocytes. Recent attempts to repeat some of Lyons' work have been unsuccessful. The problem requires further investigation, however, because of the possibility that staphylococcal capsular material exists, either in free solution or aggregated about the cell, and, because on rare occasions in the past, encapsulated staphylococci have been described.

In summary, the pathogenicity of staphylococci involves their capacity to produce toxins and their ability to become established in body tissues. Usually the pathogenic capacity of a given strain represents the total effect of both factors working together; at times one or the other is predominant. In certain generalized infections exotoxin undoubtedly is responsible for clinical manifestations of severe toxemia, and possibly contributes to the death of the individual. Staphylococcal food poisoning results directly from the ingestion of preformed enterotoxin in the food eaten. In the majority of staphylococcal infections, however, the predominating feature is invasion of the tissues and the production of a localized lesion. The local establishment of the cocci is aided by the necrotizing action of exotoxin or the destruction of leukocytes by leukocidin, but outside of the area of local involvement the effect of toxin is negligible. A small number of pathogenic staphylococci produce either no exotoxin or only an insignificant amount; infections due to these nontoxic organisms are referable to their invasive capacity. Finally, the ability of staphylococci to cause infection is conditioned by the relative susceptibility of the host and the efficiency of his defense mechanisms.

## EXPERIMENTAL INFECTION

Rabbits, mice and dogs are susceptible to experimental infection by staphylococci and to the effects of exotoxin, the rabbit being



the usual animal of choice. Guinea pigs are somewhat more resistant. The subcutaneous or intramuscular injection of even fairly large amounts of staphylococcal culture results in the formation of only a localized abscess with eventual recovery. When injected intravenously, staphylococci produce an infection which is fatal in from one to several days. Death within 24 hours is referable to the in-vivo elaboration of exotoxin. When the animals survive for several days, autopsy reveals metastatic abscesses, chiefly in the kidneys and heart and sometimes in other tissues, including bone. A fatal infection may be produced in rabbits by the intraperitoneal injection of a highly virulent strain; in mice this usually requires the simultaneous intraperitoneal injection of mucin.

Exotoxin injected intradermally into rabbits produces an intense spreading necrosis of the skin which reaches its maximum in about four days and heals slowly (Parker, 1924). The intravenous injection of exotoxin in rabbits or mice produces a characteristic series of events followed rapidly by death. For a brief interval after injection the animal appears to be normal. Then, in rapid sequence, it becomes unsteady and develops paralysis of the hind legs; the respiration, which at first is rapid, becomes irregular and gasping; in-co-ordinate running movements occur; there is incontinence of urine and feces. Death occurs after violent convulsions or, less often, the animal lies passively until death supervenes. The entire sequence often requires only from one to five minutes. Animals injected intravenously with a suspension of toxigenic staphylococci, which has been washed to remove any trace of free toxin, die after an identical terminal reaction, the only difference being that death occurs after several hours instead of a few minutes (Kleiger and Blair, 1940).

The usual laboratory animals are not susceptible to staphylococcal enterotoxin. However, intravenous injection of enterotoxin in

kittens, young cats or monkeys provokes one or more bouts of vomiting and diarrhea, beginning about one hour after its administration; the animals completely recover within 24 hours (Dolman, Wilson and Cockcroft, 1936; Dack, 1943). Food poisoning may be produced experimentally by feeding enterotoxin to monkeys or to human volunteers; the results are irregular because of individual variations in susceptibility.

### INFECTION IN MAN

Constitutional and other factors affect markedly the susceptibility of man to infection by staphylococci. Although staphylococcal infections occur in persons of all ages, they are particularly common in children and young adults; acute osteomyelitis almost characteristically occurs in the first two decades of life, while some cutaneous infections coincide approximately with physiologic changes of puberty and adolescence. Conditions such as diabetes, malnutrition or concurrent infection by other organisms, which lower the general resistance, tend to enhance susceptibility to staphylococci. Gross injury or even the minimal amount of trauma induced by friction or scratching may contribute to the establishment of conditions favoring the development of a localized cutaneous infection.

A common site of primary staphylococcal infection in man is the skin; in fact, the majority of staphylococcal septicemias and infections of the deeper tissues have their origin in cutaneous infections. Among the more common infections of the skin and subcutaneous tissues due to staphylococci are furuncles and carbuncles, folliculitis, syphilis, paronychia and many of the common suppurative infections of wounds. Staphylococci are responsible for some cases of mastitis and possibly some forms of impetigo contagiosa. In acne, staphylococci of the *albus* variety usually are found together with a diphtheroid bacillus, the latter organism being presumed to play the major

etiologic role. Furuncles, or boils, occur singly or as multiple lesions which develop simultaneously or in successive crops. Usually originating around a hair follicle, a furuncle first appears as a painful, indurated, circumscribed area of erythema which is followed in several days by the appearance of yellow pus and softening in the center of the lesion; necrosis and softening continue until eventually the pus and necrotic tissue is sloughed and replaced by granulation tissue. The development of a carbuncle is similar, but is distinguished by a lateral spreading in the deeper layers of the skin and the presence of multiple openings to the surface which discharge pus; sloughing of the necrotic tissue leaves a deep ulcer. Fever and general malaise often accompany the clinical course of a carbuncle. Cutaneous staphylococcal infections often tend to recur or become chronic. Staphylococci are found in the majority of suppurating wounds, either as the primary infecting agent or in combination with other bacteria.

Invasion of the blood stream by staphylococci results in a generalized infection which assumes either of two clinical courses. A fulminating systemic infection may occur which, it should be emphasized, is fully as severe as the septicemia due to  $\beta$ -hemolytic streptococci. It is characterized by sustained temperatures above 101° F., a pulse rate of 140 or more, leukocytosis and profound toxemia, with symptoms of irritation of both the central nervous system and the gastro-intestinal tract (Kleiger and Blair, 1940). The course is rapid and death almost invariably occurs within a few days. The more usual form of systemic infection runs a more prolonged course and is typically accompanied by the development of metastatic abscesses, often multiple, and commonly found in the lungs, kidneys, heart or bones. Endocarditis is a not uncommon complication, while in the skin small superficial abscesses or erythematous rashes occur. The responsible staphylococci may be

either toxigenic or nontoxigenic. While the symptoms of this form of generalized infection are often acute at the onset, the temperature is usually lower than in the fulminating form or is intermittent and, as the metastases develop, the clinical picture becomes that of infection of the organ involved or of disturbance of a vital function. Blood cultures, taken preferably at the height of the fever, permit differentiating the infection from other febrile diseases. In the more severe infections the cocci are present in the blood in fairly constant numbers; they are intermittently present in variable numbers in less acute conditions.

Staphylococcal septicemia originates by invasion of the blood stream from a suppurative focus. In the case of a cutaneous lesion, anatomic features at the site of the infection sometimes determine whether a septicemia will develop. For example, should staphylococci enter the rich venous plexus in the region of the nose and upper lip as a result of trauma to a furuncle, septicemia almost invariably results. Systemic infection may arise secondarily from metastatic lesions. It is common in staphylococcal, as in other pyogenic infections, to find a suppurative phlebitis or thrombophlebitis of the major veins or of venous radicles in the immediate vicinity of a suppurative lesion, arising by extension from the localized infection (Lyons, 1942). The intravascular lesion appears to be an important point of origin of systemic infections which arise from metastatic abscesses. Staphylococci are shed into the circulation from the zone of phlebitis or are carried in fragments of infected thrombus, to lodge and establish additional metastases at other sites. It has been suggested that fragments of infected thrombus are liberated into the circulation by fibrinolysin.

The mortality in systemic infections is variously reported as from 50 to 90 per cent. In the fulminating type death is almost the rule. A fatal outcome in the more common form of generalized infection is



governed in large part by the extent of metastatic involvement of vital organs or tissues and their accessibility to surgical drainage.

At least 90 per cent of all cases of osteomyelitis are caused by staphylococci. In general, the organisms are carried by the blood stream, with or without clinical evidence of their presence, from a cutaneous lesion to localize ultimately in the metaphyseal region of a long bone. The infection is manifested clinically by the sudden onset of acute pain, tenderness and local heat in the region of the metaphysis, disability of the affected part, and often re-entry of the cocci into the circulation. As the abscess develops, the pus is under considerable pressure because of the inelasticity of the bony tissue. The pus eventually finds its way to the surface of the bone where a subperiosteal abscess is formed; here it may penetrate the Haversian canals to infect the medullary cavity, or the soft tissue abscess breaks through to the surface of the skin. Direct infection of the bone may result by extension from an adjacent lesion, as occurs in osteomyelitis of the skull arising from a brain abscess. Compound fractures are infected directly from the skin or dirt incident to the trauma. When osteomyelitis becomes chronic, as it does in many instances, the local lesion, often draining pus intermittently to the surface through a sinus, persists for months or years, with periods of quiescence broken by episodes of acute exacerbation.

Staphylococcal infections of the lungs present clinical forms varying from mild tracheobronchitis to acute bronchopneumonia and the formation of multiple abscesses. Approximately 9 per cent of bronchopneumonias are due to staphylococci, the mortality often being high. Acute staphylococcal pneumonia is assumed to originate by direct infection by cocci from the upper respiratory tract. Staphylococci predominate in the friable, pinkish sputum and sometimes may be isolated in essentially pure

culture. An intense hemorrhagic infiltration characterizes the more acute forms; when less severe, multiple abscesses of varying size are formed. Empyema may be a complication. Lowered resistance as a result of some other respiratory infection often predisposes to staphylococcal pneumonia; it has been frequently observed as a sequel to epidemic influenza.

Staphylococcal infections of various other tissues or organs may be encountered. Paranephritic abscesses or multiple abscesses of the kidneys are common metastatic lesions. In the presence of renal abscesses the urine often contains blood and yields staphylococci on culture. Acute hemorrhagic nephritis is uncommon. The prostate may be the site of a primary or metastatic infection. Meningitis occurs secondarily to infection elsewhere in the body or as a result of direct infection of the membranes of the brain or spinal cord following trauma. Thrombophlebitis of the cavernous sinus arises secondarily to infections of the nasopharynx, the nasal accessory sinuses or a furuncle of the upper lip or nose. It is accompanied by severe septicemia and may be complicated by meningitis, brain abscess, osteomyelitis of the skull or orbital abscess. While puerperal sepsis is usually a hemolytic streptococcal infection, cases due to staphylococci are occasionally encountered. In rare instances, the infecting staphylococcus is strictly anaerobic.

Staphylococci are the most common cause of bacterial food poisoning (Dack, 1943). A characteristic feature of staphylococcal food poisoning is the relatively short period of incubation. The symptoms generally appear within about one to four hours after the contaminated food is eaten, in contrast to incubation periods of 12 to 24 hours or more which are typical of botulism or poisoning due to the salmonella. A brief period of nausea is followed by sudden paroxysms of vomiting accompanied by abdominal cramps, severe diarrhea and prostration. The symptoms persist for several hours;

the patient usually feels quite normal 24 hours after the beginning of the attack, although weakness or nausea may persist for a day or two. Fatalities are rare; eight deaths have been recorded in several thousand cases of staphylococcal food poisoning, and in only four could death reasonably be attributed directly to the food poisoning. The foods implicated in staphylococcal food poisoning are chiefly pastries, milk and milk products, and meats. Pastries containing custard or cream filling are frequent offenders, while a number of outbreaks have been traced to meat or meat sandwiches. The usual history is that of holding the responsible food at a relatively warm temperature for several hours before it is eaten, during which time the enterotoxin is formed. Most outbreaks due to milk have resulted from the consumption of raw milk. It has been suggested that staphylococcal enterotoxin acts on the peripheral sensory structures of the viscera rather than directly on the vomiting center in the fourth ventricle.

### DEFENSE MECHANISMS

Although the skin serves as a protective barrier against infection by many bacterial species, it is a common site of infection by the staphylococci. The healthy skin tends to free itself rapidly from organisms lodging upon it, possibly because of its slightly acid reaction or certain undefined properties of its secretions. This self-sterilizing effect removes staphylococci which are transiently present, but has little action on those which are permanent residents of the skin and which in a significant proportion are potentially pathogenic. While the pathogenesis of cutaneous staphylococcal infections is poorly understood, it is assumed that some reduction in local resistance and possibly a minimal amount of trauma provide a favorable combination of circumstances for the establishment of the local lesion.

The inflammatory reaction which follows the severe tissue damage caused by staphy-

lococci tends to produce an early walling-off of the infected area, resulting ultimately in the circumscribed lesion which is characteristic of many staphylococcal infections (Menkin, 1946). This is shown experimentally by the fact that when trypan blue is injected into an area of inflammation produced as recently as one hour earlier by the intradermal injection of staphylococci in rabbits, the dye is fixed at the site and none reaches the efferent lymphatics or tributary lymph nodes. In contrast, no fixation occurs for about six hours after the intradermal injection of Type I pneumococci, while with beta-hemolytic streptococci fixation is delayed for about two days. The readiness with which staphylococci clot blood plasma *in vitro* has suggested to some that coagulase may play a role in inducing local fixation in the tissues or in the formation of thrombi. Experiments by Menkin and others contradict this view; the exact function of coagulase *in vivo* remains problematical (Smith and Hale, 1944). However, the work by Smith et al. (1947) suggests that the initial infectivity of staphylococci for a given animal species depends largely upon their ability to clot plasma of that species, the role of coagulase being to inhibit phagocytosis of the cocci.

Phagocytosis by the polymorphonuclear neutrophils has long been recognized as an important mechanism in the defense against local staphylococcal infections. As a part of the inflammatory response, large numbers of polymorphonuclear leukocytes migrate to the site of the infection, under the chemotactic influence of the cocci and of the products liberated by the tissues. These are followed by macrophages, the sequence of cellular changes being conditioned in part by local alterations in the hydrogen ion concentration at the site of the infection. The end result of the inflammatory reaction is the formation of an abscess consisting of a central area of necrosis containing tissue debris, living or dead cocci and leukocytes, the whole sharply demarcated from



the surrounding tissue. Normally the barrier thus established prevents further extension of the infection. Needless to say, any trauma which breaks this barrier, such as squeezing a pimple or premature surgical intervention in a furuncle, may result in disseminating the infection, often with invasion of the blood stream by the cocci.

When staphylococci gain access to the circulation they tend to be removed by the cells of the reticulo-endothelial system, particularly by those in the spleen and liver (Sullivan et al., 1934). The efficiency with which the blood stream is thus cleared of cocci, especially in the presence of a more or less constant seeding from a local lesion, influences the extent and severity of many systemic infections. The blood and serum have little or no bactericidal power for pathogenic staphylococci (Spink and Vivino, 1942) and probably contribute little to the clearing mechanism. A small amount of antitoxin, usually less than 1.5 international unit, is present in most normal human sera. While this is insufficient to protect against infection, it probably modifies the picture of superficial lesions. For example, Downie (1937) has pointed out that human cutaneous lesions resemble clinically and microscopically the strictly circumscribed infection which is obtained when staphylococci are injected intradermally in a rabbit immunized with staphylococcal toxoid.

Skin reactions to staphylococcal filtrates are given by about 65 per cent of normal individuals and by nearly all persons with staphylococcal infections. No reactions are obtained in new-born infants; the proportion of positive reactors increases during the succeeding months until, at the age of about one year, the incidence of positive reactions is similar to that of normal adults. Comparable observations have been made in rabbits. Since the reaction appears to develop without relation to the antitoxin content of the blood, it has been suggested that it indicates a state of allergy. Type A polysaccharide produces an imme-

diate wheal and erythema upon intradermal injection in patients with staphylococcal infection; no reaction is given by Type B polysaccharide. The protein common to both Type A and B staphylococci produces a delayed inflammatory reaction upon similar injection.

## IMMUNITY

Heat-killed or formalinized vaccines induce in rabbits the production of agglutinins, precipitins or complement-fixing antibodies; immune sera enhance phagocytosis, but exhibit essentially no bactericidal action *in vitro*. Following the intravenous injection of staphylococci into immunized rabbits the blood stream is rapidly cleared and may be permanently freed of cocci; sterilization of the tissues is the exception although the animals may survive significantly longer than nonimmune controls (Cowan, 1939b). There is no clear relation between the presence of antibacterial antibodies and protection against the cocci; there is, in fact, some evidence that the observed protection is in part nonspecific (Cowan, 1939b). Lyons (1937) suggests that protection is due to a specific "anti-capsular" antibody, which can be prepared by immunizing rabbits with young (3-hour), heat-killed encapsulated cocci. This antibody is stated to agglutinate young cocci, sensitize encapsulated cocci for phagocytosis and prevent the destruction of leukocytes; when staphylococci are injected intravenously into a rabbit so immunized, the blood is rapidly cleared and sometimes sterilized.

The immunization of rabbits with toxoid or toxin produces high titers of circulating antitoxin (Burnet, 1929; Downie, 1937). The immunity thus obtained is antitoxic and not antibacterial. Animals are protected against the immediate necrotic or lethal effects of the cocci or of toxin, and outlive controls when challenged with a lethal intravenous dose of living cocci, but meta-

static abscesses ultimately develop. Cutaneous lesions produced by the intradermal injection of staphylococci in toxin-immune rabbits show a minimum amount of necrosis and are less extensive than those produced in controls; staphylococci can be recovered in cultures from these lesions for about one week after injection. However, when rabbits are immunized with a heat-killed suspension of staphylococci which has been washed to remove any trace of free toxin, the intradermal injection of staphylococci results in a cutaneous lesion which is indistinguishable from that produced in controls (Downie, 1937). Valentine (1936) suggests that toxoid for immunization should contain leukocidin as well as exotoxin. Downie's rabbits which were immunized with toxoid owed some of their immunity to antileukocidin. Active phagocytosis was a prominent feature of the minimal cutaneous lesions in these animals, but no phagocytosis was observed in the animals immunized with washed (toxin-free) vaccine. Forssman (1938) described a type of immunity which could not be ascribed to any of the known antibodies since it reaches its height after agglutinins and other antibacterial antibodies have disappeared and is not related to antitoxin. Several attempts by others to reproduce Forssman's results have been unsuccessful (e.g., Downie, 1937).

Following spontaneous staphylococcal infection in man, the agglutinins are increased. In superficial infections the titer of circulating antitoxin rises only slightly or not at all, while in osteomyelitis it may or may not be high. A spontaneous staphylococcal infection confers little or no protection against either recurrence or subsequent infection.

Serum obtained by immunizing animals with staphylococcal vaccine has some prophylactic value when given shortly before an infecting intravenous dose of cocci but has no therapeutic effect once the infection has become established. As in the case of

active immunity, the life of experimental animals may be prolonged beyond that of controls, but sterilization of the tissues and the prevention of metastatic abscesses is not consistently obtained. Type A antibacterial serum, which precipitates the specific polysaccharide and is stated to contain no antitoxin, confers protection irregularly against cutaneous or generalized infection in animals. Antitoxic serum protects against the immediate lethal effect of toxin and against the development of cutaneous lesions in rabbits by either the cocci or toxin. Downie's results on active immunity with washed vaccine and with toxoid were paralleled by similar results when sera from these animals were used for passive protection. Some of the protective effect of antitoxin is undoubtedly referable to its content of antileukocidin. Passive protection conferred by antitoxin does not prevent the development of metastatic abscesses; once the individual is enabled by antitoxin to overcome the initial period of toxicity, the clinical picture becomes that resulting from invasion by the staphylococci.

## DIAGNOSIS

Staphylococci are readily cultured from pus, blood and other pathologic material on the usual meat-infusion media. When freshly isolated, colonies of pathogenic staphylococci vary in shade from deep gold to pale cream; less often white colonies may be encountered. For the best production of pigment the medium should contain a carbohydrate, preferably glucose, lactose or mannitol. A zone of hemolysis may surround colonies on the surface of blood agar; plate hemolysis per se is significant only in relation to other properties and does not necessarily indicate that a culture is toxigenic. Material from patients who are receiving sulfonamide or penicillin therapy should be cultured on media containing para-amino-benzoic acid or penicillinase, respectively. The characteristic growth on agar, supple-



mented by the examination of Gram-stained smears, readily identifies a staphylococcus. Food poisoning staphylococci exhibit the same cultural reactions as other pathogenic strains. Since staphylococci are often present in many foods, significance can be given to their isolation from a suspected food only when they are present in very large numbers. The demonstration of the production of enterotoxin requires the injection of culture filtrates into kittens or monkeys.

An important problem in laboratory diagnosis is the differentiation of pathogenic from nonpathogenic staphylococci. Considerable weight has been given in the past to pigmentation, hemolysis on blood agar, fermentation of mannitol and liquefaction of gelatin, and culture media employing these reactions have been proposed for the isolation and identification of pathogenic staphylococci (Chapman, 1944). There is little question that freshly isolated staphylococci from pathologic sources tend to exhibit all or most of these properties which can thus serve as presumptive evidence of pathogenicity. It must be kept in mind, however, that individual strains vary considerably and that nonpathogenic varieties exhibit some of the same reactions, although usually to a lesser degree. The coagulase test, on the other hand, constitutes the most reliable, single in-vitro criterium for the identification of pathogenic staphylococci and the cultural properties only support the evidence supplied by it. In the absence of a standard method of performing the coagulase test the following technic is given:

A young agar or broth culture is used. To a serologic tube containing 0.5 cc. of fresh citrated rabbit plasma, diluted 1:5 with broth, is added an equal volume of a broth culture or one loopful of growth from agar. The cocci are thoroughly suspended in the plasma, and the mixture is incubated in the water bath at 37° C. As controls, a tube inoculated with a known coagulase-positive staphylococcus and a tube of uninoculated plasma are in-

cluded. The tubes are examined for clotting at intervals over a period of three hours. Nearly all strains of pathogenic staphylococci clot plasma within this time, many of them within one hour or less. Since a small proportion of strains require a longer period of incubation, tubes which show no clotting at three hours are incubated for an additional 18 hours and are examined at convenient intervals. The clot produced varies from one which is solid and immovable when the tube is inverted to a loose clot or occasionally only a trace of fibrin. Any degree of clotting is regarded as a positive reaction. There appears to be no relation between the amount of clotting and the severity of the infection from which the staphylococcus is isolated. Details of the technic may be varied somewhat without materially affecting the reaction. Thus, either citrated or oxalated rabbit or human plasma is satisfactory, and the plasma may be used undiluted or diluted as much as 1:10.

Using the known fact that staphylococci are rapidly clumped by plasma, Cadness-Graves and her associates (1943) have devised a "slide test" for the identification of pathogenic strains. It is reliable, rapid and applicable to the routine testing of cultures and to surveys in which large numbers of colonies on plates are examined directly.

Cocci from a suspected colony are evenly suspended in a drop of water on a slide and a drop of plasma is thoroughly mixed with the suspension by continuous stirring. Coagulase-positive staphylococci are clumped in large irregular masses within 30 seconds or less; nonpathogens are clumped only after two minutes or more. The delayed clumping of nonpathogens appears to be due to natural agglutinins in the plasma. Rapid clumping of coagulase-positive staphylococci is also obtained with fibrinogen solution.

The use of plasma-agar or fibrinogen-agar plates for testing the production of coagulase has been proposed, an opaque zone being formed around colonies of coagulase-positive staphylococci. The evaluation of this method must await further investigation, for other staphylococcal products may sometimes produce opacity on plasma agar.

## TREATMENT

Numerous biologic and chemical agents have been employed in the past for the treatment of staphylococcal infections, but none has proven entirely satisfactory. Antibiotics, particularly penicillin, are the most satisfactory at the present time.

Undoubted benefit has sometimes resulted from vaccine therapy, particularly with autogenous vaccines, in the treatment of chronic or recurring cutaneous staphylococcal infections. However, vaccine therapy has not completely fulfilled the hopes raised by their introduction 40 years ago, and the reported failures are nearly as numerous as the successes. Staphylococcal vaccines often contain a small amount of toxoid; it is not unlikely that some of the beneficial effects, both experimentally and in man, come from the antitoxic immunity which they induce.

While localized staphylococcal infections appear at times to be benefited by bacteriophage therapy, the results in septicemia are unconvincing, and, as a rule, the mortality rate is not significantly reduced. Although this form of therapy has had its proponents in recent years, it appears to be the general consensus that little is to be gained from the use of bacteriophage.

Staphylococcal toxoid is of greatest value in the treatment of subacute or chronic cutaneous infections and has generally given far more successful results than vaccines. Clinical improvement is accompanied by an increased titer of circulating antitoxin; the titer obtained varies with the individual, and there is no definite antitoxin level of the blood which can be said to demarcate successful therapy from failure. The immunity derived is antitoxic; while it persists, the tissues are protected against the necrotic effects of the toxin and normal reparative processes are enhanced, but no antibacterial effect is obtained. As measured by freedom from recurrences, protection by toxoid therapy for periods of a few months to two years has been claimed. Recurrences

are usually characterized by milder lesions which often respond to a second course of toxoid therapy. In a series of approximately 3,000 cases of cutaneous staphylococcal infections treated with toxoid over a ten-year period, Ramon and his associates (1946) recorded failure in only about seven per cent.

Staphylococcal antitoxin has a definite but restricted therapeutic place in fulminating systemic infections with evidence of severe toxemia. When administered intravenously early and in adequate amounts, antitoxin may carry the patient over the acute emergent period (Dolman, 1934; Kleiger and Blair, 1943). It must not be forgotten that after survival of the acute phase of the infection the clinical picture becomes chiefly that of the effects of metastases which are so characteristic of invasion of the blood stream by staphylococci. Antitoxin therapy is of little value in this phase, nor can it be expected to influence an infection due to a nontoxigenic strain.

In a series of about 100 cases of staphylococcal septicemia the potential value of Julianelle's Type A antibacterial serum was suggested by an apparent reduction in the incidence of metastatic lesions; however, the mortality rate was not significantly lowered (Julianelle, 1942).

Sulfonamide drugs, sulfathiazole and sulfadiazine in particular (Henderson, 1946), aid in preventing the spread of an established local infection and thus reduce the possibility of septicemia. When used prophylactically, however, they do not appear to prevent the development of infection and, on the whole, they are less effective against staphylococcal infections than against those caused by more susceptible organisms.

Penicillin is effective in the treatment of the majority of superficial, systemic or deep-seated staphylococcal infections. Adequate amounts often shorten the course of an acute infection and reduce the chances of complications or of fatalities. The literature contains frequent reference to the response to penicillin of infections which had



been refractory to sulfonamides or other forms of therapy. The incidence of toxic reactions is low. Larger doses of penicillin are ordinarily required for staphylococcal than for other types of infection, and the response to therapy is governed in part by the nature and site of the infection. As a rule, fresh infections in well-vascularized soft tissues or localized infections which can be drained readily respond well to penicillin therapy, even in the presence of a severe systemic infection. The antibiotic has proved to be a valuable adjunct in the management of surgical infections. In chronic osteomyelitis the systemic and local administration of penicillin permits the primary closure of a wound after adequate operative treatment (Buchman and Blair, 1945). Surgical infections due to staphylococci often present collections of thick pus or lesions surrounded by relatively avascular fibrosed tissue into which penicillin does not penetrate readily from the blood; these conditions demand that the systemic administration of penicillin be supplemented by its local injection and frequently by surgical treatment. It cannot be emphasized too strongly that in any condition in which sound surgical judgment indicates the necessity for operation the complementary use of penicillin does not alter the general principles of surgery. Antibiotics and chemotherapeutic agents provide a useful aid to therapy but do not supplant adequate surgical intervention. Failure of penicillin therapy may be traced to such factors as inadequate dosage, overwhelming infection, use of the drug in the terminal stages of the infection, inadequate surgical treatment, or the presence of staphylococci which are naturally resistant or have acquired resistance to the drug during treatment. Mixed infections with Gram-negative bacilli are more difficult than those due to staphylococci alone. In the presence of a penicillin-resistant strain of staphylococci recourse may be necessary to some other

antibiotic, to a sulfonamide drug, or, in their proper sphere, to toxoid or antitoxin.

Tyrothricin is a moderately effective therapeutic agent in a limited number of staphylococcal infections (Henderson, 1946; Goldman et al., 1947). Since this antibiotic cannot be administered systemically, its chief value lies in the local treatment of such lesions as infected wounds or ulcers. On the whole, unless an infection is due to an especially susceptible strain, staphylococcal infections are somewhat less amenable to treatment with tyrothricin than those caused, for example, by  $\beta$ -hemolytic streptococci. The antibiotic is ineffective in wounds which are grossly contaminated with Gram-negative bacilli. Tyrothricin may serve as a useful substitute for penicillin in the local treatment of lesions due to a penicillin-resistant staphylococcus.

## EPIDEMIOLOGY AND CONTROL

Some individuals are constant carriers of potentially pathogenic staphylococci on the skin or in the nose, and it is commonly assumed that the staphylococci which the patient himself harbors often are responsible for his infection. As pointed out above, invasion of the blood stream and the subsequent deep-seated infections frequently originate in a cutaneous lesion. It is conceivable, but difficult to prove, that the cocci present on the skin, in hair follicles or other sites require only the combination of a lowered local tissue resistance and possibly a minimal trauma in order to initiate an infection. In fact, there is accumulating evidence, based on the identification of staphylococci by specific slide agglutination with absorbed sera or by bacteriophage typing, that staphylococci isolated from a patient's skin or nose are often identical with the organisms responsible for his infection. Traumatic or surgical wounds may obviously become infected by cocci which are carried into the wound from the skin, on particles of dirt, or by droplet in-

fection from the nasopharynx of the attendant at the time dressings are being changed. The direct transmission of staphylococcal infection is probably rare.

In cases of food poisoning the incriminated food is often found to have been prepared or handled by an individual who is a nasal or skin carrier of pathogenic staphylococci, or who has an open staphylococcal lesion on the hands, arm or face. Reports of outbreaks of staphylococcal food poisoning show that not infrequently after the food is prepared it is held at room temperature for several hours before being served, thus providing a period of incubation during which the enterotoxin is formed. The ubiquity of staphylococcal carriers makes it impracticable to eliminate them from handling food. Individuals with obvious cutaneous infections should, of course, be excluded from the preparation or handling of food. Pasteurization of pastries, or reheating cream puffs or éclairs for a brief period after they have been filled with custard, when properly carried out, reduces staphylococcus contamination and does not affect flavor or appearance. The simplest and most reliable method of control involves the refrigeration of every food substance which may be a potential source of food poisoning.

### GAFFKYA TETRAGENA

These organisms occur as spherical cells, typically occurring in body fluids in groups of four; they are Gram positive, facultatively anaerobic and grow slowly on artificial culture media. They are parasitic upon mucous membranes of the respiratory tract. The type species is *Gaffkya tetragena* (Gaffky) Trevisan.

The distinguishing feature of these cocci is their arrangement in tetrads. This grouping is always seen in material taken directly

from the body, while in cultures the cocci are arranged in fours or pairs. In pus or body fluids the tetrads are surrounded by a wide capsulelike halo which is not always demonstrable in cultures. The individual cocci are about 0.8 micron in diameter. On agar the colonies are grayish-white, less opaque and somewhat smaller than those of the staphylococci, and are adherent and viscid. Variants occur which show smooth or mucoid, yellow, pink or brown colonies. Several carbohydrates are fermented; gelatin is not liquefied. Originally known as *Micrococcus tetragenus*, these organisms form a separate genus of the family *Micrococcaceae* (Bergey's Manual, 1948).

The pathogenicity of *Gaffkya tetragena* for mice is high, and is characterized by a rapidly fatal septicemia with metastases in various organs. Rabbits and guinea pigs are less susceptible to infection.

Relatively few human infections due to *Gaffkya tetragena* have been reported. Reimann (1935) believes that they are probably more common than suspected. Infection usually takes place only when the host's resistance is lowered by some predisposing condition; a common precursor is a respiratory infection. Young adults appear to be particularly susceptible. The cocci produce abscesses of the soft tissues, arthritis, meningitis, pneumonia and empyema. A penile ulcer due to an anaerobic form has been described recently. The course of the infection is from mild to severe and is accompanied by a remittent fever and leukocytosis. Several instances of septicemia have been reported, some of which were fatal. A nonfatal case of septicemia with purulent arthritis and meningitis was reported by Reimann (1935).

Treatment with vaccine has given variable results. The successful use of penicillin in sepsis due to *Gaffkya tetragena* has been reported.



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# 14

## The Anthrax Bacillus

### INTRODUCTION

*Bacillus anthracis* is a large aerobic sporulating Gram-positive micro-organism, typically occurring in the form of chains of rod-shaped cells. It is the only species of the genus consistently virulent for animal and man. The disease in man is primarily a skin infection which frequently progresses to a fatal septicemia. It also occurs as a pulmonary form but only infrequently (Fig. 1E and J, Fig. 2E).

### HISTORY

Although anthrax has long been known as a disease of animals and man, accurate diagnosis of this infection was not possible until its etiology was definitely established by Robert Koch in 1877. Hence, many of the early reports on its occurrence must be viewed as somewhat speculative. Prior to Koch's etiologic studies, the condition of the animals at necropsy suggested the names "splenic fever," "charbon," and "milzbrand" for the disease. In man it was usually referred to as "malignant pustule" or "malignant carbuncle," whereas the occasional pneumonia came to be known as "wool sorter's disease" or "rag sorter's disease" because of the industrial groups most often affected by this manifestation of the infection in the nineteenth century.

*B. anthracis* was the first organism proven to be the causative agent of an infectious disease. Davaine and Rayer, in 1850, and Pollender, in 1855, presented evidence that

rod-shaped organisms were present in animals infected with anthrax. Robert Koch saw bacteria in the blood of animals dead of the disease, obtained pure cultures, produced fatal infections in laboratory animals by inoculation with these cultures, and reisolated the bacterium from the experimental infections. This procedure established *B. anthracis* as a pathogen beyond reasonable doubt.

### MORPHOLOGY

The anthrax organism tends to form chains of rod-shaped cells with an appearance sometimes compared to a bamboo pole. Undue diagnostic emphasis is often placed on the square ends of *B. anthracis*. Its cell size and shape vary with the strain, medium and the age of the culture, or with the conditions of lesions from which it is recovered. In general, the cell is from 0.8  $\mu$  to 1.0  $\mu$  in width and from 2 to 4  $\mu$  in length. Chains of more than 20 cells are often obtained in liquid cultures. Spores are formed by the fusion of "sporogenic" granules. Within the cell, the spore occupies a median position. Motility is never observed. This is an important differential point in the separation of *B. anthracis* from the nonpathogenic spore-forming aerobes, many of which are motile. Although *B. anthracis* is typically Gram positive, there are found occasional Gram-negative cells which are particularly

conspicuous when they occur in a chain of Gram-positive organisms.

Smears from infected tissues usually reveal encapsulated forms of *B. anthracis*, but their occurrence in cultures is more variable. Preisz (1911) pointed out that certain types of capsules, large and "viscous," do not contribute to virulence as do the "firm" capsules. These morphologic observations invite chemical study of the different types of capsules produced by *B. anthracis*, with a view toward a better understanding of the relationships between virulence and capsule formation.

### CULTURAL CHARACTERISTICS

*B. anthracis* is a fairly strict aerobe. Spore formation depends even more than growth on free oxygen. To insure an adequate supply of oxygen, the organisms can be grown on an agar slant or other supporting base, in flasks placed on a shaking machine and agitated 30 to 60 times per minute, or sterile air can be bubbled through liquid cultures at the rate of one-half to two volumes of air for each volume of medium per minute.

The nutritional requirements of *B. anthracis* have been determined by Gladstone (1939) and by Brewer et al. (1946) with somewhat different conclusions, perhaps because different strains were used by the two groups of workers. According to Brewer et al., thiamine was the only vitamin required, but the addition of uracil, adenine or guanine to the basal medium stimulated growth. Gladstone did not find it necessary to add these components to get maximum growth, although he reported that glutamine was essential. The mineral requirements for good growth include calcium, magnesium, iron, manganese, potassium, bicarbonate and phosphate. Spore counts as high as  $10^9$  per milliliter of culture were obtained with the "synthetic" medium used in Brewer's laboratory.

Growth characteristics on various media have been employed as identifying criteria, although virulence provides a more certain means of identification. Growth on sheep, human or rabbit blood agar is usually without hemolysis in contrast to the hemolysis noted around colonies of saprophytic spore forming aerobes. However, certain strains of the anthrax organisms do produce a small



FIG. 19. Impression smear of the edge of colony of anthrax bacillus grown on the surface of gelatin medium; stained with eosin methylene blue decolorized with eosin.  $250\times$ . (Zettnow, E., 1902, Atlas Photographischer Tafeln nach Originalaufnahmen, in Kolle, W., and Wassermann, A., Handbuch der pathogenen Mikroorganismen. Jena, Fischer, plate 9, illustration 213.)

zone of hemolysis, and some avirulent spore forming aerobes do not. On a plain agar surface, convention decrees that the anthrax bacillus shall produce a round colony, having a cuneiform or cut-glass appearance when viewed with transmitted light, but many other organisms, particularly those of the *B. subtilis* group, also give this type of colony. Furthermore, variants of *B. anthracis* give quite different types of colonies. Similarly, the slow liquefaction of gelatin, with growth in stabs resembling an "inverted fir tree," is of no real value in identifying *B. anthracis*.



## RESISTANCE

Disinfection requires the use of physical or chemical agents lethal to the anthrax spore. A five-minute exposure to 100° C. will kill wet spores of *B. anthracis* as does autoclaving, provided there is adequate heat transfer. It must be remembered that large flasks of cultures or large contaminated articles offer serious handicaps to the transfer of heat and thus require longer periods for disinfection. Anthrax spores resist desiccation for years. A common laboratory procedure for preserving virulent strains of this organism is to mix sporulating cultures with dry, sterile earth. Freezing has little effect on the viability of either the spore or vegetative forms. However, both vegetative and spore forms are gradually destroyed by storage in the moist state at temperatures appreciably above freezing.

Iodine and chlorine are most effective in destroying spores and vegetative cells. On the other hand, mercurials, phenolic compounds and detergents are more bacteriostatic than bactericidal against *B. anthracis*, and surviving organisms can often be recovered if the chemical is adequately removed from the environment and from the cell surface.

## VARIATION

Variations in virulence and spore formation were first noted in Pasteur's laboratory (1883). Changes in colony form and other properties have since been described by various workers (Preis, 1911; Wagner, 1920; Nungester, 1929; Sterne, 1937). In general, the more virulent forms give rise to rough colonies. Smooth, mucoid and phantom colonies have been noted as well as numerous intermediate colony forms. It is probably correct to state that there is no absolute relationship between colony form and virulence. However, a significant difference in the virulence of variants of *B. anthracis* has been recently established by introducing the bacteria into the lungs of

animals, but no difference could be detected by subcutaneous inoculation.

Spores of a given strain of *B. anthracis* usually give rise to cultures with the same properties as those of the parent vegetative cells, even after storage for long periods of time in the spore state. This fact is of considerable value in preserving unstable properties of strains such as virulence. Living-spore vaccines of various grades of virulence, used for the immunization of animals, are obtained by preparing the spore suspensions from vegetative cells having the desired virulence.

## PATHOGENICITY

Anthrax is primarily a disease of sheep, cattle, horses and swine. Man contracts the disease infrequently. There were only 1,683 human cases reported in the United States in the 20 years ending in 1938. The disease is found more or less throughout the world, wherever there are susceptible animals. However, certain areas are more affected than others, as, for example, parts of France and the delta of the Mississippi River. Mice and guinea pigs are the most susceptible of the common laboratory animals. Infections can readily be produced in rabbits, hamsters and monkeys. Nonfatal infections usually result from the inoculation of even large doses into pigs and dogs. As a rule, rats are also quite resistant to infection with *B. anthracis*.

In man, anthrax is primarily a skin infection, which is described in some detail in the section on diagnosis. The pulmonary form of the disease, although frequently referred to, is of rare occurrence. In a personal communication, Dr. Herman Gold of Philadelphia states that he has treated one pulmonary and 73 cutaneous anthrax infections over a period of years. This experience contrasts with the early reports of "rag-sorter's disease" and "wool-sorter's disease" which were assumed to occur fairly frequently. Extensive experiments on various

laboratory animals exposed to aerosols of anthrax spores led Young et al. (1946) to conclude that, although a fatal septicemia might result from such exposure, the disease was not characterized by pneumonia.

In animals, the type of disease varies with the species affected and the site of inoculation. Since most farm animals contract the disease by ingestion of contaminated food, the signs are referable to the gastro-intestinal tract, with a terminal septicemia. In sheep, the signs may be mistaken for those of malignant edema. Marked gelatinous edema characterizes the more severe cutaneous forms of the disease (Fig. 20), and severe congestion of blood vessels of the viscera is found at autopsy of animals with fatal infections. This latter finding has given rise to the names charbon and splenic fever.

The pathogenesis of the disease is not fully explained. There is often evidence of a local lymphatic spread of the bacterium once the infection starts. Ultimately, at least in the more severe experimental or spontaneous infections, there is a bacteremia. The accumulation of bacteria in the blood stream is usually not marked until shortly before death. It is reasonable to assume that the fixed phagocytes of the liver, bone marrow, and lymphoid tissue are responsible for removing bacteria from the blood during the preterminal stages of the disease. Tremendous numbers of bacteria are present after death in the tissues containing fixed phagocytic cells. The fact that only vegetative cells are found in the infected animal has been explained on the basis that the presence of calcium ions and lack of adequate free oxygen in the body are conditions which prevent sporulation.

Recent studies on the pathogenesis of experimental anthrax (Watson et al., 1947; Cromartie et al., 1947; Bloom et al., 1947) have extended the earlier work of Bail (1929), who postulated that *B. anthracis* produces in infected animals a factor which he termed "aggressin" and which either

stimulates immunity or lowers resistance depending on whether it is given in advance of or simultaneously with a challenging inoculation of the pathogen.

A chemical and biologic investigation was made by Watson et al. of the inflammatory exudate produced at the site of subcutaneous inoculation in animals. Using biochemical procedures selected to produce minimal alteration of labile chemical sub-



FIG. 20. Photograph showing a *B. anthracis* lesion in skin of rabbit 72 hours after injection of spores. (Cromartie, Watson, Bloom and Heckly, 1947, Studies on infection with *Bacillus anthracis*. II. The immunological and tissue damaging properties of extracts prepared from lesions of *B. anthracis* infection. Journal of Infectious Diseases, 80, 15.)

stances, a separation of two significant components from the exudate was accomplished. One of these fractions, a polypeptide containing a large amount of d(-) glutamic acid, resembled one previously isolated by Ivánovics et al. (1937) from *B. anthracis*. The biologic properties of the polypeptide isolated by Watson et al. (1947) are noteworthy. Injected subcutaneously, it elicits a histologic reaction similar to that seen in anthrax. It greatly increases clotting time in a system employing purified fibrinogen. These biologic properties could not be demonstrated with the glutamyl polypeptide obtained from *B. subtilis*. Furthermore, material with similar biologic activity was not obtained from *B. anthracis* grown



in vitro in ordinary media. A second factor isolated from the edematous exudate was a protein which stimulated a solid immunity in rabbits. This protein was destroyed in 45 minutes at 57° C. It was also partially inactivated by precipitation with ammonium sulphate. No substance with similar biochemical or immunizing properties has yet been obtained from cultures of *B. anthracis* grown in ordinary media.

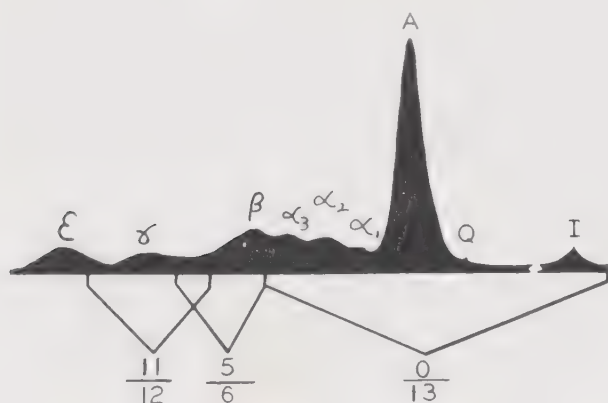


CHART 8. Electrophoretic pattern of extract from anthrax lesion showing the relative position of normal serum components, tissue damaging factor (I) and protective antigen which is masked by normal globulins of the rabbit. (Watson, D. W., Cromartie, W. J., Bloom, W. L., Kegeles, G., and Heckly, R. J., 1947, Studies on infection with *Bacillus anthracis*. III. Chemical and immunological properties of the protective antigen in crude extracts of skin lesions of *B. anthracis*. Journal of Infectious Diseases, 80, 31.)

The results of an electrophoretic analysis of the crude anthrax tissue extract are shown in Chart 8. The usual serum albumin and globulin components are in evidence. The rapidly moving (strongly electronegative) component marked "I" is the polypeptide (T.D.F.) or tissue damaging factor. The immunizing fraction is masked by the serum globulins. However, the immunizing properties of this portion of the exudate (to be referred to later) is clearly indicated by the data from immunization experiments included in the chart. The nu-

merator of the fraction represents the number of rabbits which survived and the denominator the number inoculated with a challenge dose of *B. anthracis* after immunization with the electrophoretically separated portion of the anthrax exudate.

The cause of death in anthrax is still uncertain. One of the less objectionable theories is the blocking of blood capillaries by clumps of bacilli. However, if interference with blood circulation and resulting cardiovascular collapse seems an attractive hypothesis, it might be desirable to look for factors other than the organisms themselves as interfering with blood flow. An interference with circulation of blood by changes in the surface of the red blood cells is known to occur in other infectious diseases.

Changes in the blood and tissue chemistry of rabbits infected with *B. anthracis* have been studied by Bloom et al. (1947), who reported a hyperglycemia in the fatal infections and not in immunized animals which recovered. This suggests an effect on the adrenals which is possibly a common result of severe infections or even of trauma in general. There also appeared to be a disturbance of calcium metabolism since administration of this electrolyte saved 50 per cent of 10 animals, whereas all untreated controls died. The authors suggest that the terminal episodes of anthrax present similarity with magnesium poisoning. The sedimentation rate increased as would be expected, and the number of mature polymorphonuclear leukocytes dropped rapidly in the fatally infected rabbits. In the immune animals there occurred a slight rise in mature polymorphonuclear leukocytes, followed by a drop which, however, did not reach the low level seen in the animals with fatal infections.

The bacterial count varied from  $2.3 \times 10^2$  to  $3.5 \times 10^7$  organisms per milliliter of blood on the day of death. The fact that some animals died with as few as 2 or 3 bacteria per milliliter of blood tends to discount the

"plugged capillary" theory as the cause of death.

### NATURAL RESISTANCE

The response of animals to anthrax varies from more or less complete resistance to high susceptibility, as in the case of the mouse. Chickens and rats possess high resistance, while sheep and rabbits are quite susceptible. Man's resistance is probably intermediate. Resistance also varies within species. Certain breeds of sheep have been introduced for animal husbandry in Europe because of their higher resistance to anthrax infection.

The basis for natural resistance to anthrax has been studied periodically ever since Pasteur postulated that the higher body temperature of chickens contributed to their resistance. On this assumption, he subjected chickens to cold, lowered their body temperature and reduced in effect their resistance to infection with *B. anthracis*. Wagner (1890), however, questioned the validity of the assumption by pointing out that *B. anthracis* grows satisfactorily at 43° C. in the blood and serum of fowls, in vitro. The multiple effects of lowered temperature on the physiologic mechanisms of the host and their relation to resistance remain obscure. Although it is now known that a decrease in phagocytic activity was one factor effected by chilling of the fowl, there may be still other unknown mechanisms. The resistance of the rat to anthrax was attributed by von Behring to the germicidal power of its blood serum. Yet the rabbit's serum is also bactericidal despite the fact that this animal is susceptible to anthrax. Cromartie et al. (1947) have found that in susceptible animals, such as guinea pigs and rabbits, the organisms proliferate at the site of injection and remain encapsulated and stain well with Giemsa stain throughout the progressing infection; few leukocytes accumulate around the bacteria. The picture is different when the bacteria

are injected into a resistant animal, such as the rat. They grow normally for about four hours. Then, following a large infiltration of polymorphonuclear leukocytes, the capsules of the bacteria disappear, and their cytoplasm lose the ability to take Giemsa

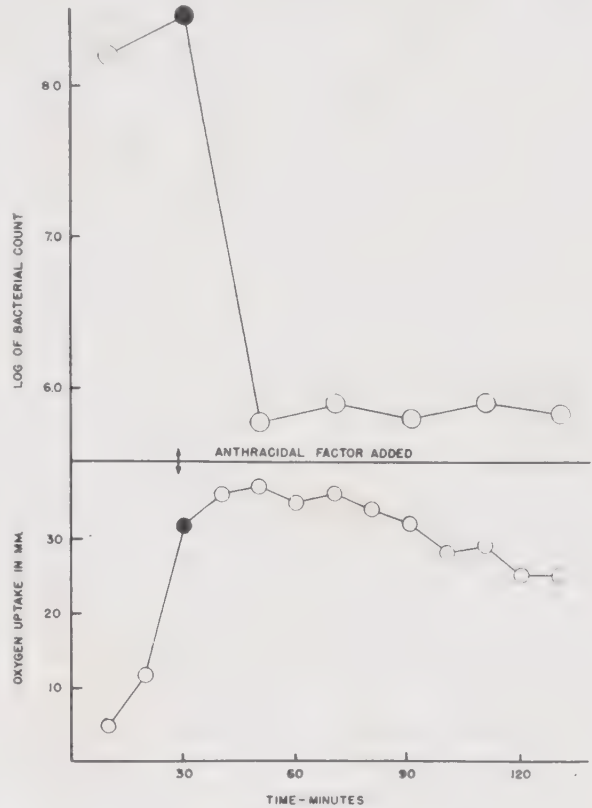


CHART 9. Correlation between oxygen uptake as measured in the Warburg respirometer and the anthracidal power of extract of normal rabbit cecum. (Bloom, W. L., Watson, D. W., Cromartie, W. J., and Freed, M., 1947, Studies on infection with *Bacillus anthracis*. IV. Preparation and characterization of an anthracidal substance from various animal tissues. *Journal of Infectious Diseases*, 80, 46.)

stain; the organisms finally disintegrate and disappear. Actual phagocytosis is not conspicuous, and the anthracidal effect appears to be due to tissue fluids arising in part possibly from the leukocytes.

Subsequent experimental work by Bloom et al. (1947) led to the isolation of a bactericidal substance from dog leukocytes (thus confirming and extending the earlier



findings of Hawkin and of Vaughan and McClintock), and to the recognition of a similar anthracidal agent in hog pancreas, calf thymus and rabbit cecum. Chemical and electrophoretic mobility studies suggest that the fraction from rabbit cecum is a basic polypeptide; it inhibits the respiration of anthrax cells and kills them (Chart 9). Some protection against anthrax is obtained by treating infected animals with this polypeptide during the infection.

Resistance to anthrax is also related to various nonspecific defense mechanisms which are active in protecting the host against pathogens in general. The covering epithelium is a most important defense. The very location of malignant pustules is often determined by the trauma of certain areas of the skin such as the back of the neck of stevedores who carry contaminated hides on their shoulders. Fascial planes aid in limiting the spread of the lesion.

### IMMUNITY

The classic experiment made by Pasteur at Pouilly-le-Fort in 1881 stands as a milestone in the history of infectious diseases. In this experiment 24 sheep, 1 goat, and 6 cows were vaccinated with living, attenuated anthrax cultures which had been grown at 42° to 43° C. in broth for two to three months. The animals received two inoculations 12 days apart, the second vaccine being less attenuated than the first one. Two weeks after the second vaccination all animals, and the same number of controls, were infected with a fully virulent culture of *B. anthracis*. All but one of the vaccinated animals survived, while all of the control sheep and goats died of anthrax. The one sheep which died in the vaccinated group did not have anthrax. A severe infection developed in the control cows while the vaccinated animals were fully protected. Although the results of this fortunate experiment clearly indicate the feasibility of producing active immunity with attenuated

organisms, subsequent experience with Pasteurian vaccines has been, for some unknown reason, far less satisfactory. At the present time, a variety of vaccines is in use. The Pasteurian vaccines consist of a series composed of the "premier vaccin" made of vegetative living forms attenuated by growing at 42° to 43° C. for two or three weeks; a somewhat less attenuated culture is used to make the "deuxième vaccin." Not only are different cultures now used to prepare the living vaccine but, in some preparations, spores from attenuated strains are employed. Passive immunization has also been combined with active by Sobernheim to permit the use of a less attenuated antigen.

The efficacy of anthrax vaccines as measured by challenging sheep at time intervals of from 4 to 360 days after immunization has been reported by Gochenour, et al. (1935). In the group of sheep vaccinated with the bacterial vaccine, 35 per cent survived when challenged at 4 days, 100 per cent at 16 days, 17 per cent at 108 days; 25 per cent of the nonvaccinated controls survived. Better results were obtained when the sheep were vaccinated with an anthrax spore vaccine. About 65 per cent of the animals so vaccinated, and challenged four days after vaccination, survived. All these immunized animals survived if challenged 16 days after vaccination. Although the data indicated that some protection is supplied by various vaccines if the challenge dose is small, it is clear that this low degree of protection is not sufficient.

In 1905, Bail suggested the use of extracts of infected tissue as immunizing agent, and others have since confirmed that such extracts confer some immunity to animals. Watson et al. (1947) have recently isolated and studied a protective antigen from an extract of the subcutaneous edema of infected rabbits. In separating the antigenic material, care was exercised to avoid reagents and procedures which might destroy a labile substance. Manipulations were car-

ried out in the cold. Ethanol, instead of ammonium sulphate, was used for protein precipitation. The Tiselius-electrophoresis apparatus was employed to check fractionating procedures and also for actual electrophoretic separation of components of the tissue extract. The antigen isolated appeared to be a protein and was destroyed by heating at 57° C. for 30 minutes and by several proteolytic enzymes including one from *B. anthracis* itself. Rabbits immunized with this antigen resisted a challenge of over 100 lethal doses of anthrax spores. Other animals, however, such as mice, hamsters and guinea pigs received much less protection from immunization with the same protein. Why this antigen should be so much more effective in the species from which it was obtained is indeed an interesting and perplexing problem.

It is interesting to note that Grabar and Staub (1946) succeeded in immunizing guinea pigs with a fraction separated from edema fluid and containing one of the anthrax polysaccharides previously recognized by Ivánovics (1939). Gladstone (1946) has reported the production in vitro of an antigen apparently similar to that obtained from edema fluid, by growing anthrax bacilli in media containing bicarbonate and a protein fraction from plasma. Although injection of this antigen did not lead to the production of demonstrable antibodies it induced in sheep and other animals protection against many lethal infective doses of anthrax.

Immune serum is sometimes injected into animals together with attenuated, living cultures of anthrax as insurance against the accidental production of disease during active immunization. It can be, although rarely is, used prophylactically. In experimental animals, solid protection can be afforded by the use of adequate doses of antisera. Protection lasts only from seven to ten days. Finally, antiserum was widely used for the treatment of established infection before the advent of the more effective

chemotherapeutic agents. In event of infection with a drug-resistant organism, such therapy would definitely be in order. It is reasonable to assume that, had not the chemotherapeutic agents proven so effective, additional study of the preparation and use of antisera would have improved this fundamental therapeutic procedure.

## DIAGNOSIS

A history disclosing possible exposure is of great aid to the physician, since he might not even consider such an infrequently occurring disease in a differential diagnosis. The cutaneous lesion begins with a small red macule which enlarges, and, after two or three days, local edema may be present. A vesicle filled with clear fluid occupies a central position on the macule and is soon followed by satellite vesicles. The organism may be easily demonstrated in the early lesion. The lesion becomes necrotic, and the black eschar present in the older lesions is characteristic of anthrax. The cutaneous lesion is not painful, but tenderness of regional lymph nodes does occur. Malaise, fever and general prostration develop in keeping with the severity of the disease. The local edema may also become quite marked in the more severe infections.

Detection of the organism is best done by inoculating mice with material from the lesion, blood, tissues or articles suspected of being contaminated; this technic tends to screen out nonpathogenic contaminants. On death of the animal, blood-agar plates can be streaked, and a Giemsa-stained smear of the spleen can be examined. The blood-agar plate inoculated from the mouse should show gray, roughened, nonhemolytic colonies in contrast to similar colonies with a zone of hemolysis produced by nonpathogenic forms of the spore-forming aerobes. Occasionally some strains of *B. anthracis* show slight hemolysis. Carbohydrate fermentation is variable and of little value for identification.



Direct microscopic examinations of material from a cutaneous vesicle may reveal the Gram-positive rods and permit a tentative diagnosis which is strengthened if the organisms are nonmotile. It should be mentioned however, that nonvirulent, nonmotile bacteria having many of the characteristics of *B. anthracis*, and referred to as *B. anthracoides*, are occasionally encountered in tissues and may lead to erroneous bacteriologic diagnosis. On the other hand, it is well to bear in mind that putrefying carcasses of animals dead of anthrax may fail to yield positive cultures on account of the lethal effects of products of putrefaction, such as hydrogen sulphide, on the anthrax organism.

Extracts of infected tissues, prepared by boiling and filtering, show a ring of precipitate when carefully layered over immune serum. The reaction, probably due to a heat stable polysaccharide, which has been isolated by Schockaert (1929) from animals dying of anthrax, is known as the Ascoli test, and has been used extensively in detecting anthrax in carcasses. Good antisera and appropriate controls with extracts of normal tissue are essential. Agglutination tests with *B. anthracis* are not satisfactory, due to the instability of the antigen suspensions.

### TREATMENT

At the present time, the treatment of choice is the administration of penicillin in total doses as large as 4 or 5 million units, if necessary. Probably 1 or 2 million units divided into 30,000 units every three hours until bacteria have disappeared from the lesion and clinical indications warrant would be the usual treatment. Ellingson et al. (1946) treated 25 cases of cutaneous anthrax by this method with no deaths. Although the series is small, the effectiveness of penicillin treatment appears convincing when the results in individual patients are considered. Sulfadiazine has been used alone or in combination with penicillin in a few

cases. Animal experiments indicate that it should be effective.

Untreated victims of cutaneous anthrax have an expected case fatality of about 20 per cent. It has generally been accepted that most, if not all, patients with "wool-sorter's disease" die of the infection. The effect of modern chemotherapy on this rare form of the disease is yet to be determined.

### EPIDEMIOLOGY

Anthrax is found throughout the world with the possible exception of the frozen waste lands of the Arctic and Antarctic. Since the spores remain viable in soil almost indefinitely, an area once contaminated will continue to have at least sporadic cases for years to come. Certain areas exhibiting a perennial occurrence of the disease in animals have come to be known as "anthrax districts." In the United States, southeastern South Dakota, northeastern Nebraska, part of the Texas Gulf coast and the lower Mississippi river valley from Arkansas to and including the delta region are particularly affected (Stein, 1942).

Anthrax in farm animals is more frequent during the dry season of late summer and fall. Ponds, marshes and lowlands become available for grazing at that time, and the animals probably have an increased chance of taking in contaminated soil with close grazing. Floods also increase the occurrence of anthrax in animals.

The disease in man occurs in agricultural areas, and more usually in manufacturing districts where animal products, such as hides and hairs, are processed. In this country, the tanneries of the Philadelphia, Boston and New York areas supply many of the human cases. The use of items such as shaving brushes or furs made from contaminated animal products may result in occasional disease in man. Anthrax, however, is not a highly contagious disease among human beings. Only about 80 cases a year occur in the United States, and sec-

ondary cases are rare. The apparent prevalence of "wool sorter's disease" at an earlier period, compared to its rarity in recent years, cannot be explained with certainty. It is possible that improved sanitation of factories and general improvement in handling animal products is partly responsible for the apparent reduction in pulmonary anthrax. Skin abrasions are probably the most important predisposing factor favoring infection. Age is of minor importance. The disease is more frequent in males simply because they are more likely to come in contact with the organisms. The large number of laboratory infections recently reported from Camp Detrick (Ellingson et al., 1946) is due to the fact that extensive work with highly virulent strains was in progress at the time these infections occurred.

### CONTROL MEASURES

Accurate diagnosis of the disease in animals followed by treatment or disposal of the carcass by burning or deep burial are essential steps in a control program. Disinfection of contaminated ground with lye and change of pastures are indicated following an outbreak in grazing animals. Barriers to keep blood-sucking flies, dogs, rats, coyotes, and particularly buzzards, away

from diseased animals or their excreta should be set up since they probably disseminate infectious material.

Animal products should be decontaminated as soon as possible during processing so as to minimize exposure to workers. General procedures to insure clean, well-ventilated working quarters in industries subject to anthrax are of help because dust may be a factor in transmitting the disease to man. Since abrasions of the skin are significant as a predisposing factor, an effort should be made to reduce skin injuries by the use of mechanical aids in handling heavy items and by wearing gloves when feasible. Emphasis should be placed on adequate "first aid" treatment of all skin abrasions of those likely to be exposed. Tincture of iodine, 2 per cent, should be used on scratches.

Active immunization of susceptible domestic animals has been extensively practised with at least some success in decreasing the incidence of this disease. Because no satisfactory nonliving vaccine is available, immunization of human beings is impractical. Since the incidence of the disease in man following exposure is apparently low, passive immunization or chemotherapeutic agents have not been employed for prophylactic purposes.

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# 15

## The Clostridia

### INTRODUCTION

Organisms of the genus *Clostridium* are anaerobic or microaerophilic rods, producing endospores which in some species are wider than the vegetative rods from which they arise. They are generally Gram positive. Many species, but not all, decompose proteins or ferment carbohydrates, and many form exotoxins. About half the derived species are pathogenic.

The natural habitats of the clostridia are the soil and the intestinal tract of higher animals and man. They are widely distributed in all parts of the world. The 50 recognized species may be roughly divided into four groups.

(1) *Saprophytes*—About 25 species; many are active decomposition organisms, especially in the soil; a few, as *C. acetobutylicum*, are used in industrial fermentations. (2) *Gas gangrene group*—5 or 6 species are responsible for gas gangrene; another 8 or 10 species are frequently associated with wound infections. (3) *C. tetani*—the causative agent of tetanus. (4) *C. botulinum*—the causative agent of botulism (Fig. 1F, G and H).

### ANAEROBIOSIS

In 1861, in the course of work on butyric acid fermentation, Pasteur found a large bacillus which failed to grow in an atmosphere of ordinary air but grew luxuriantly and produced butyric acid in the absence of oxygen. Pasteur introduced the terms aerobes to designate organisms which require oxygen and anaerobes for organisms

which normally grow in the absence of air. Somewhat later the term micro-aerophil was introduced to describe organisms which are less exacting than obligate anaerobes but require for growth a lower oxygen tension than atmospheric air. There is a wide difference in oxygen tolerance among species of *Clostridium*. McLeod (1930) for example, demonstrated that *C. tetani* will grow on the surface of blood agar in air up to 5 to 15 mm. of mercury, while *C. perfringens* will grow in air at 200 mm. of mercury and grow very slowly up to half an atmosphere air pressure. These two species represent approximately the extremes in the group.

Several explanations have been offered for the inability of anaerobes to utilize oxygen and for their intolerance toward oxygen (see page 27). (1) All anaerobic forms examined lack cytochrome, cytochrome oxidase, catalase and peroxidase enzyme systems. (2) McLeod (1923) advanced the thesis that all bacteria can form hydrogen peroxide on exposure to oxygen. In the case of anaerobes which lack catalase and peroxidase,  $H_2O_2$  tends to accumulate to toxic concentrations in the presence of oxygen. (3) It has been postulated that obligate anaerobes can carry out their metabolic reaction only at a negative oxidation-reduction potential. If a chemically adequate medium is well aerated, the oxidation-reduction potential will be strongly positive: from +0.2 to



+0.3 volts in terms of Eh. Aerobes will grow in such medium, but not obligate anaerobes. If the medium is deaerated by boiling, rapidly cooled and protected from air, the Eh will become more negative (+0.01 to -0.1 volts), and many aerobes will grow. The addition of a reducing sugar or of more effective reducing agents, such as cysteine, ascorbic acid, sodium thioglycollate, can reduce the Eh further and all anaerobes, both obligate and facultative, will grow provided the medium is in other respects adequate (Hewitt, 1936; Reed and Orr, 1943).

### MORPHOLOGY AND CULTIVATION

All species of *Clostridium* consist of relatively large Gram-positive rods, though in old cultures some Gram-negative forms are often found. All, by definition, produce spores. In most species the spores are greater in diameter than the rods in which they are formed. Some, like *C. perfringens*, rarely sporulate; most others sporulate freely. A few species, like *C. perfringens*, produce capsules; the majority do not. Most species possess peritrichous flagella and are actively motile.

With proper anaerobic conditions, most clostridia grow readily in a variety of organic media. Anaerobic conditions can be conveniently obtained by incubating cultures in the McIntosh-Fildes type of anaerobic jar, in which air is first displaced by hydrogen and the residual oxygen removed by combination with hydrogen in the presence of colloidal platinum. Following isolation, cultivation of pure cultures is conveniently obtained in fluid media to which is added chopped muscle or about 0.1 per cent agar and a reducing agent (as sodium thioglycollate) or, where permissible, a reducing sugar. Cultures in such media in deep tubes may be handled freely exposed to air.

### ISOLATION AND IDENTIFICATION

The following procedure will generally be found adequate for the isolation and primary identification of pathogenic and associated species (Reed and Orr, 1941).

Wound exudates, tissue fragments, or other material, should be examined after Gram staining. Gram-positive rods in wound exudates always suggest the presence of *Clostridium*, but negative results are inconclusive.

(1) Plate directly on blood agar and egg-yolk agar and incubate aerobically and anaerobically for 24 hours, or, if time is not pressing, for 48 hours. (2) Inoculate at the same time several tubes of chopped meat media with the material under examination. Heat some of the tubes, after inoculation, to 70° C. for 20 minutes. Incubate for 24 hours. Plate from these cultures as under (1). This is desirable even when the primary plates show well-defined colonies. (3) Examine plates from (a) and (b) in detail; a colony microscope is indispensable. Table 32A summarizes the types of colonies, the reactions in blood and egg-yolk media and the morphology of the organisms likely to be encountered. In many instances it will be possible to fish colonies directly; it will frequently be necessary to replate. The greatest difficulty will result from spreading forms. This is largely obviated by using plating media with a relatively dry surface and by adding a small amount of a dessicant to the bottom of the anaerobic jar. (4) Once pure cultures have been obtained, subcultures may be made in the necessary media for the reactions summarized in Table 32B. The results from these reactions will ordinarily permit tentative identification. Final identification of pathogenic forms depends upon the production of toxin and its neutralization by specific antitoxin. (See the following sections of this chapter.)

The materials used in these tests are prepared as follows:

**Egg Medium** for lecithinase action. Collect the yolk of an egg aseptically and mix with an equal volume of sterile 0.85 per cent NaCl. Add 10 ml. per 100 ml. of agar base which has been sterilized and cooled to 50° C. Mix and pour into plates.

**Chopped Meat Medium.** Add 200 grams ground heart to 1 liter of infusion broth, boil 5 minutes, adjust to pH 7.6, boil another 5 minutes and readjust to pH 7.6. Dispense

in deep tubes to give a depth of about half an inch of meat particles and 2 inches of fluid.

**Media for Biochemical Reactions.** To infusion broth, as used for blood agar, add enough agar and sodium thioglycollate to final concentrations of 0.1 per cent. This may be used as a base for fermentation, or for indole or nitrite reactions. For H<sub>2</sub>S reactions the same medium may be used with 0.02 per

TABLE 32A. COLONY FORM, REACTIONS ON BLOOD AND EGG AGAR AND MORPHOLOGY OF PATHOGENIC AND RELATED SPECIES OF *Clostridium*

	COLONY FORM *	ZONE OF HAEMOLYSIS	EGG PPT. ZONE **	RODS †	SPORES ‡
<i>C. tetani</i> .....	F	+	D	S	STC
<i>C. botulinum</i> .....	B	+	B	T	OEC
Gas gangrene group					
a. <i>Toxigenic sp.</i>					
<i>C. perfringens</i> .....	A	+ (double)	A	TC	OE rare
<i>C. novyi</i> .....	D	+	B	S to T	OEC
<i>C. septicum</i> .....	D	+	D	S to T	OEC
<i>C. bifermentans</i> .....	D	+	A	S to T	OEC
<i>C. histolyticum</i> .....	C	+	D	S to T	OEC
b. <i>Associated sp.</i>					
<i>C. fallax</i> .....	D	+		S to T	OE rare
<i>C. tertium</i> .....	D	+	D	S to T	OEC
<i>C. sporogenes</i> .....	D	±	C	T	OEC
<i>C. multifementans</i> .....	A	—		T	OEC
<i>C. aerofetidium</i> .....	A	—	D	T	OE rare
c. <i>Rare sp.</i>					
<i>C. carnis</i> .....	B	+	D	T	OTC
<i>C. sphenoides</i> .....	B	+	D	T	STC rare
<i>C. tetanomorphum</i> .....	E	±	D	S to T	STC
<i>C. difficile</i> .....	B	+	D	S to T	OEC
<i>C. capitovialis</i> .....	C	+	D	S to T	OTC
<i>C. cochlearium</i> .....	E	+	D	T	OTC

\* Colony forms on blood agar:

- A. Large raised colonies, smooth to slightly ridged with entire to undulate margins, 2 to 4 mm. in diameter
- B. Smaller raised colonies smooth to irregular with entire to undulate or serrate margins, 1 to 3 mm. in diameter.
- C. Minute colonies, raised, smooth to irregular with entire to irregular margins with short rhizoids, 0.2 to 1 mm.
- D. Large colonies, raised, very irregular, with wide spreading coarse rhizoids, 3 to 6 mm. diameter.
- E. As D, but smaller with finer rhizoids, 1 to 2 mm. in diameter.
- F. Irregular granular colonies with delicate spreading rhizoids to irregular spreading rhizoidlike structures without a definite central colony.

\*\* Colony forms on egg-yolk agar:

- A. Wide zone, 6 to 10 mm., of precipitation extending well beyond the colony. No luster.
- B. Narrow zone, 2 to 4 mm., of precipitation beyond the colony. Iridescent luster over colony and precipitation zone.
- C. Precipitation only under colony. Iridescent luster over colony.
- D. No precipitation, no luster.

† Morphology: S—slender rods; T—thick rods; C—capsulated.

‡ Spores: Rare—rarely formed; T—terminal; C—central; E—excentric; S—spherical; O—oval; C—*Clostridium*



TABLE 32B. BIOCHEMICAL REACTIONS OF PATHOGENIC AND RELATED SPECIES OF *Clostridium*

SPECIES	MILK	DEX-TROSE	MALT-OSE	LAC-TOSE	SALI-CIN	SU-CROSE	HYDRO-GEN SULFIDE	GELA-TIN LIQUE-FAC-TION	NI-TRATE RE-DUC-TION	INDOL	MILK AGAR DI-GES-TION
<i>C. perfringens</i> .....	Stormy	+	+	+	—	+	+	+	+	—	—
<i>C. butyricum</i> (group)....	Stormy	+	+	+	+	+	+	+	+	—	—
<i>C. multi fermentans</i> .....	Stormy	+	+	+	+	+	—	—	+	—	—
<i>C. aerofoetidum</i> .....	Stormy	+	+	+	+	—	+	+	+	—	—
<i>C. tertium</i> .....	Acid	+	+	+	+	+	+	—	+	—	—
<i>C. fallax</i> .....	Acid	+	+	+	+	+	+	—	+	—	—
<i>C. paraputrificum</i> .....	Acid	+	+	+	+	+	±	—	—	—	—
<i>C. carnis</i> .....	Acid	+	+	+	+	+	—	—	—	—	—
<i>C. chauvoei</i> .....	Acid	+	+	+	—	+	+	+	+	—	—
<i>C. septicum</i> .....	Acid	+	+	+	+	—	+	+	+	—	—
<i>C. sphenoides</i> .....	Acid	+	+	+	+	—	+	—	—	+	—
<i>C. botulinum</i> .....	Acid	+	+	—	?	—	+	+	—	—	—
<i>C. parabolulinum</i> .....	Digested	+	+	—	?	—	+	+	—	—	+
<i>C. novyi (oedematiens)</i> ....	Digested	+	+	—	—	—	+	+	—	—	±
<i>C. bifermentans</i> .....	Digested	+	+	—	—	—	+	+	—	+	+
<i>C. sordellii</i> .....	Digested	+	+	—	—	—	+	+	—	+	+
<i>C. sporogenes</i> .....	Digested	+	+	—	—	—	+	+	—	—	+
<i>C. histolyticum</i> .....	Digested	—	—	—	—	—	+	+	—	—	+
<i>C. tetanomorphum</i> .....	No change	+	+	—	—	—	±	—	—	±	—
<i>C. difficile</i> .....	No change	+	—	—	+	—	—	—	—	—	—
<i>C. capitolalis</i> .....	No change	+	—	—	—	—	±	—	—	+	—
<i>C. cochlearium</i> .....	No change	—	—	—	—	—	—	—	—	—	—
<i>C. tetani</i> .....	No change	—	—	—	—	—	+	±	—	—	—

cent of lead acetate, but without thioglycolate.

**Gelatin.** Infusion broth with 5 per cent gelatin.

**Protein Digestion.** To the same infusion broth add from 5 to 8 per cent fresh egg white or from 8 to 10 per cent blood serum. Mix in a Waring blender for a few seconds, allow the foam to be absorbed by standing overnight in the cold. Tube in deep tubes and autoclave.

**Milk.** Add 50 grams of dry skim milk powder to 1,000 ml. infusion broth, adjust to pH 6.8, dispense in deep tubes with 0.1 gr. reduced iron and autoclave.

GAS GANGRENE

INTRODUCTION AND HISTORY

In 1607 Fabricus Hildanus (Kellett, 1939) recorded the earliest known case of gas gangrene with such clarity that later descrip-

tions have not added greatly to clinical knowledge of the disease.

... the wheel of the cart tore up the whole of the inner part of the leg. The periosteum was ripped from the tibia for a palm's length ... having washed the wound with red wine and tepid water ... I enveloped the whole of the leg in a bandage soaked in vinegar ... He had a restless night ...

On the 4th day ... I undid the dressings and found ... the whole of the outer part of the leg and the foot itself mortified and covered with large black vesicles containing water similar to that in which meat had been washed ... at a certain place I could make out a sound as if there were some sort of empty space underneath. Inferring, therefore, that the disease was present underneath in that place I began to explain ... that amputation was clearly going to prove fruitless ... that evening a vesicle the size of an egg arose in his groin ... and within the next two hours the scrotum became swollen the size of a head and gangrenous. At about

the third hour after midnight he became covered with sweat, at first hot then cold, and he died peacefully, in the middle of a sentence, four days, eleven hours after his illness commenced.

Hildanus, an experienced observer, regarded this case as a great rarity and, as far as can be judged from the historical records, the disease remained rare until the eighteenth century. It was not until the Napoleonic wars that gas gangrene became a common disease of the wounded. With succeeding European wars it occurred with increasing frequency although it was relatively rare during the Crimean war and the American Civil war. The disease probably reached the highest peak in the early part of World War I when some ten per cent of the wounded developed gas gangrene.

Pasteur and Joubert (1877) isolated from putrid sheep blood a pathogenic anaerobic bacillus which they called *Vibrion septique*. Four years later Koch (1881) discovered what he regarded as the same bacterial species; in 1884 Chaveau and Arloing came to the conclusion that gas gangrene is a clinical entity caused by Pasteur's organism (now called *C. septicum*). Welch and Nuttall (1892) isolated from gas gangrene wounds a very different organism which they called *Bacillus aerogenes capsulatus* (now *C. perfringens*) and Novy (1894) described a third species which he called *Bacillus oedematiens* No. 2 (now *C. novyi*). Hence, at the beginning of World War I three organisms had been described as the causal agents of gas gangrene. The many cases among the wounded in France from 1914 to 1918 provided much of the material on which modern knowledge is based (Weinberg and Séguin, 1918).

#### SPECIES OF CLOSTRIDIA CONCERNED IN GAS GANGRENE

The following species of toxin-forming clostridia are now regarded as the primary cause of gas gangrene: *C. perfringens* (syn.

*C. welchii*); *C. novyi* (syn. *C. oedematiens*); *C. septicum* (syn. *Vibrion septique* or *B. oedematis maligni*); *C. bifermentans* (syn. *C. sordellii*; the latter is a toxigenic variety of the former). In about 40 per cent of the cases only one of these is present in the wound, whereas one or more of the above toxigenic forms, together with one or more nontoxigenic species as *C. sporogenes*, *C. fallax*, *C. tertium*, etc., occur in 60 per cent of the cases (Table 33A). The frequency of

TABLE 33A. PROPORTION OF CASES OF GAS GANGRENE IN WHICH A SINGLE SPECIES OR MORE THAN ONE SPECIES OF *Clostridium* WAS PRESENT

SPECIES OF <i>Clostridium</i>	WEINBERG (1918) 91 CASES	MACLENNAN (1944) 146 CASES
	<i>per cent</i>	<i>per cent</i>
1 species only		
<i>C. perfringens</i> .....	32	34
<i>C. novyi</i> .....	5	17
<i>C. septicum</i> .....	1	5
<i>C. bifermentans</i> .....	..	0.7
<i>C. histolyticum</i> .....	..	0.7
<i>C. fallax</i> .....	1	..
<i>C. aerofœtidum</i> .....	1	..
More than one species..	60	42

occurrence of the more important species is summarized in Table 33B. In rare instances, other species of *Clostridium* are present as the primary agent (Table 32A).

TABLE 33B. INCIDENCE OF *Clostridium* IN GAS GANGRENE WOUNDS

SPECIES	FRANCE, 1914-1918 234 CASES	NORTH AFRICA 146 CASES
	<i>per cent</i>	<i>per cent</i>
<i>C. perfringens</i> .....	65	57
<i>C. novyi</i> .....	20	38
<i>C. septicum</i> .....	13	20
<i>C. bifermentans</i> .....	..	5
<i>C. fallax</i> .....	6	14
<i>C. histolyticum</i> .....	2	7
<i>C. sporogenes</i> .....	11	?



The gas gangrene and related species of *Clostridium* are widely distributed. If a gram or two of garden soil is shaken up in a tube of broth, heated to 70° C. for 20 minutes and plated out anaerobically, one to several gas-gangrene species will be recovered from almost every sample. In a

In the case of *C. septicum*, Henderson (1934) was able to divide the species into four groups on the basis of O antigens and to subdivide the groups on the basis of H antigens. *C. perfringens* cultures are agglutinated by homologous antisera, but, for the most part, heterologous strains are not agglutinated (Orr and Reed, 1940).

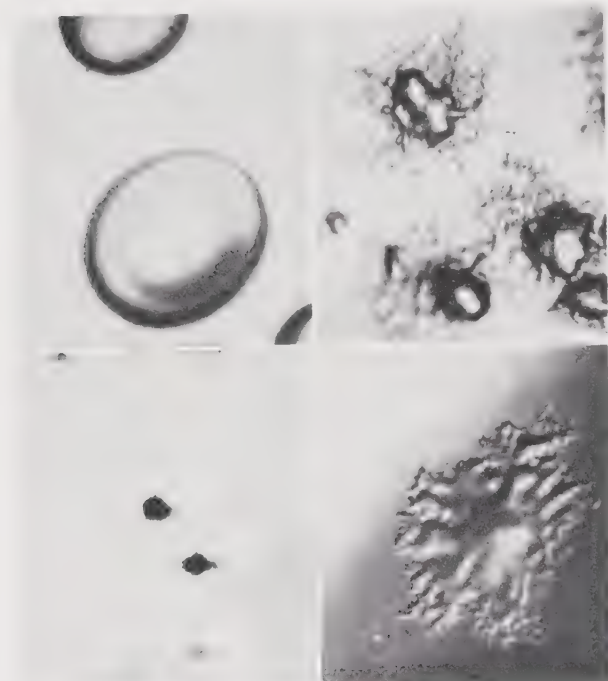


FIG. 21. Colonies of *Clostridium*: 48-hour growth on blood agar in an anaerobic jar. (Top, left) *C. perfringens*, (top, right) *C. novyi*, (bottom, left) *C. histolyticum*, (bottom, right) *C. sporogenes*.

study of 200 samples of European soils *C. perfringens* was recovered from 100 per cent, *C. novyi* from 45 per cent, *C. septicum* from 8 per cent and *C. histolyticum* from 2 per cent.

The Clostridia isolated from soil or wounds vary greatly in virulence. They follow the familiar pattern of variation in colony structure with accompanying differences in antigenic structure and general virulence (Orr et al., 1933). Their antigenic structure is complex (McCoy and McClung, 1938). Motile species, which include most members of the gas gangrene group with the conspicuous exception of *C. perfringens*, contain H and O agglutininogens.

#### FACTORS WHICH INFLUENCE INFECTION

Gas gangrene most frequently develops in wounds or compound fractures in which there has been much shattering of tissue,

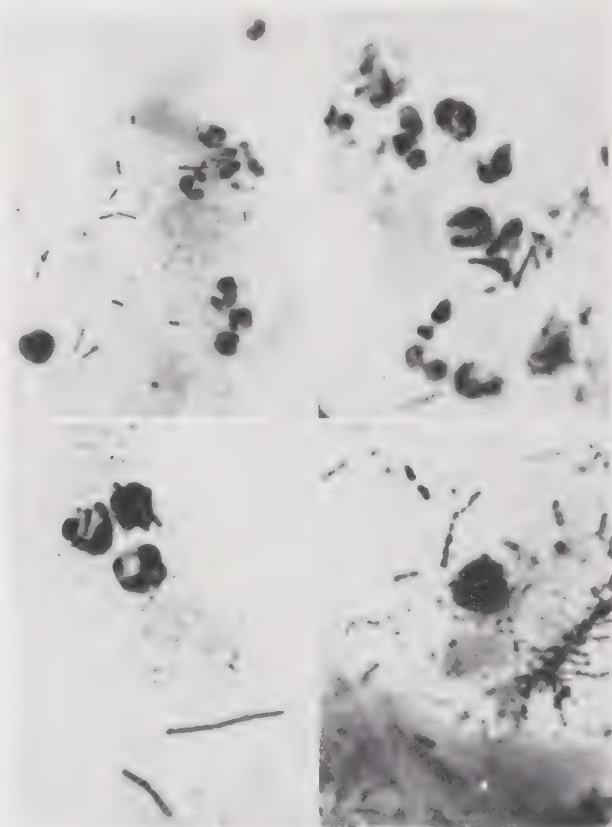


FIG. 22. Films of exudate from gas gangrene wounds stained by Gram's method.

along with the introduction of soil, or other foreign matter. It is therefore to be expected that, at least in the first stages, a variety of organisms will be present. In addition to one or more species of *Clostridium*, various cocci and *Proteus* types are common. Even in well-established cases of gas gangrene

the mixed infection not infrequently persists.

The mere presence of even toxigenic species of clostridia does not necessarily lead to gangrene. MacLennan (1943) recognized three clinical conditions in the wounded in North Africa: simple contamination with *Clostridium* which occurred at some stage in the history of 20 to 30 per cent of the wounded; of these some 5 per cent developed a relatively mild "anaerobic cellulitis" while only 1.5 per cent (i.e., 0.34 per cent of the total number of wounded developed true gas gangrene. Physical conditions of the wound and the general virulence of the organisms introduced determine the development and nature of the infection. Anaerobic conditions are essential for spore germination and for growth which is promoted by the presence of partly or completely devitalized tissue and by foreign bodies as bone splinters, soil or other material. Soil is particularly important, as small amounts of calcium salts tend to lower the oxidation-reduction potential of tissues (Fildes, 1929).

#### ENZYMES AND TOXINS

Spreading infection which characterizes gas gangrene is associated with the production of certain enzymes by the developing organisms. Most of the invasive species produce fibrinolytic enzymes (Reed et al., 1943) and hyaluronidase, which depolymerizes hyaluronic acid, the substance mainly responsible for cementing together tissue

cells (McClellan, 1943). Some species produce collagenase, which causes breakdown of connective tissue elements (Oakley et al., 1946). Finally, the principal lethal toxin of *C. perfringens*, and to a lesser extent of other species of the group, is itself an enzyme, lecithinase.

Bull and Pritchett (1917) demonstrated the presence of a soluble toxin in filtrates of *C. perfringens* cultures and that the toxin is neutralized by a specific antitoxin. Since that time the five most important species of the gas gangrene group, *C. perfringens*, *C. novyi*, *C. septicum*, *C. bifermentans* and *C. histolyticum*, have been shown to produce specific soluble toxins neutralized by their respective antitoxins. All these toxins exhibit hemolytic, lethal and necrotizing actions. These several actions appear in some cases to be different manifestations of a single substance but in others to be due to different toxins.

It was long supposed that all strains of *C. perfringens* produced an identical toxin neutralized by a single antitoxin. Study of a series of diseases of sheep brought to light a group of organisms with most of the characteristics of *C. perfringens* except for the nature and specificity of the toxins produced. Accordingly the species has been divided into four types, A, B, C, and D, which produce seven distinguishable toxins listed in Table 34 (Oakley, 1943). Type A is the only one so far recovered from human infections. The  $\alpha$  toxin which it produces is a lecithinase (Negler, 1939; Macfarlane and Knight, 1941) and its lethal

TABLE 34. TOXINS PRODUCED BY FOUR TYPES OF *C. perfringens*

BACTERIAL TYPE	TOXIN						
	$\alpha$	$\beta$	$\gamma$	$\delta$	$\epsilon$	$\eta$	$\theta$
A	+++	-	-	-	-	+	+
B	+	+++	+	+	++	?	?
C	+	+++	+	++	-	?	?
D	+	-	-	-	+++	?	?



action is proportional to the rate at which it splits lecithin to phosphocholine and a diglyceride. *C. perfringens*  $\alpha$  toxin is now ordinarily assayed by determining its lecithinase action, either using egg yolk or purified lecithin as substrate (Zamecnik et al., 1947). The  $\theta$  toxin, which is antigenically distinct from  $\alpha$  toxin, produces similar hemolytic lethal and necrotizing effects, but it is not a lecithinase.

*C. septicum* toxin exerts hemolytic, necrotic and lethal effects, but these appear to be different manifestations of one single toxin. There is some lecithinase action, but it is not proportional to the lethal action. *C. novyi* and *C. bifermentans* toxins exhibit some hemotoxic but much more potent necrotic and lethal effects. There is also a lecithinase action, disproportionate to the lethal action. *C. histolyticum* culture filtrates contain a weak toxin. Other species ordinarily associated with gas gangrene do not, as far as we are aware, produce toxin.

#### PATHOLOGY

The earliest effect of the infection is a rapidly spreading edema involving primarily subcutaneous connective tissue and, a little later, connective tissue of muscle bundles. Collagen fibers are swollen and fragmented and the cellular elements of the connective tissue largely broken down. Gas bubbles appear early as an accompaniment of edema. Blood vessels in the connective tissue or muscles are thrombosed and frequently the endothelium is ruptured. These changes may be seen in both the area where organisms are proliferating and in a zone beyond where no organisms can be found. At this stage, fragmenting connective tissue may be adjacent to apparently healthy muscle. Later, destruction of muscle occurs and the organisms may spread in a muscle from the area of the wound toward the extremities. At the site of the wound the muscles may be black, friable and diffuent; further out, the blackened

area is generally succeeded by a deep red zone and sometimes a yellow band of discoloration adjacent to healthy tissue. Gas appears first as bubbles between muscle fibers and later in the surrounding areolar tissue. As the gas increases, the tissues yield a tympanic note on percussion and the skin usually acquires a dusty hue. At times, however, gangrene of deep muscles may be far advanced without visible changes in the skin (Reports on Anaerobic Bacteria and Infections, 1919; Coupal, 1929). In a strictly localized gas gangrene the only physical sign may be a foul-smelling discharge containing gas bubbles.

The physical signs and gross pathology of infections caused by the different species of *Clostridium* do not differ widely. In *C. novyi*, *C. septicum* and *C. bifermentans* infections there is a rather more copious accumulation of blood-stained mucoid material in the subcutaneous tissues and between muscles than in *C. perfringens* infections. The exudates tend to be more foul smelling, and local tissues appear to undergo a somewhat more rapid destruction, where the infections are complicated by the presence of proteolytic species like *C. sporogenes* or *C. histolyticum*.

#### PROPHYLAXIS AND TREATMENT

All the toxins of the gas gangrene group give rise to specific antitoxins on injection into suitable animals, and *C. perfringens*, *C. novyi* and *C. septicum* antitoxins have been widely used in therapy. The antitoxins are prepared, usually in horses, by the customary methods of immunization initially with toxoids and finally with toxins or whole cultures. The refined and concentrated antiserum globulins are standardized in terms of international units of specific antitoxin. The League of Nations standards of gas gangrene antitoxins are measured in terms of the weight of specified standard toxin neutralized.

Where antitoxin therapy is indicated the

urgency is ordinarily too great to delay treatment until a bacteriologic diagnosis is made. On the other hand, while the clinical evidence of the presence of gas gangrene is clear, the differences in the symptoms produced by the several species are indefinite. The practice therefore is to give a polyvalent antitoxin. A frequent procedure, where the serum is used for prophylaxis, is to give intravenously or intramuscularly 9,000 units *C. perfringens*, 4,500 units *C. septicum* and 3,000 units *C. novyi* antitoxins. Once the disease has become established 3 times this dosage is ordinarily advised, repeated every 4 to 6 hours, depending upon the response of the patient. In a fairly representative series of severe gas gangrene cases, 81 per cent of 25 untreated cases died as against 51 per cent of 114 treated with antitoxin (Macfarlane, 1943). It should be noted, that, in this series, evidence of benefit from antitoxin therapy was apparent only in cases in which the patient received adequate surgical treatment (see Hall, 1945, for a comprehensive review of the protective value of antitoxins in experimental animals).

The chief problem in the preparation of gas-gangrene toxoids is to obtain toxins of high potency. Detoxification with formalin and precipitation with alum is carried out by the same methods used in the preparation of diphtheria and tetanus toxoids (Stewart, 1942). *C. perfringens*, *C. septicum* and *C. novyi* toxoids induce an antitoxic immunity in guinea pigs, rabbits and man (Tytell et al., 1947). The antitoxin levels attained appear to be sufficient to be of great prophylactic value but there are no actual data of exposure of immunized persons on which to base conclusions.

All the gas gangrene anaerobes are sensitive to sulfonamides in vitro (Reed et al., 1944), and acute infections in experimental animals respond to treatment with various sulfonamide preparations (Reed and Orr, 1941; Bliss et al., 1941). Reed and Orr found that in the case of gas-gangrene

wounds in guinea pigs a much larger percentage of the animals recovered when the drug was introduced directly into the wound than when it was given orally. When administered locally the drug reaches higher concentrations in infected tissues than when introduced via the blood stream, especially in the case of fragments of tissue cut off from their blood supply. Penicillin appears to be more effective than any of the sulfonamides (Hac, 1944). There is much evidence that combined chemotherapy and antitoxin therapy is superior to either alone (Siebenmann and Plummer, 1945). It should be emphasized that chemotherapy is not a cure for gas gangrene but at best only a supplement to antitoxic and surgical treatment.

## TETANUS

### INTRODUCTION AND HISTORY

Tetanus is a toxemia due to infection of wounded or otherwise injured tissues by *Clostridium tetani*. The disease is characterized by convulsive tonic contraction of voluntary muscles. In human cases the first symptoms are generally muscular spasms in the region of the local infection followed by triismus, which rapidly increases to fixation. Other muscles of the neck, arms, trunk and legs become progressively involved until all the voluntary muscles of the body are in a state of tonic spasm. In some cases the toxemia remains localized near the site of infection.

The disease has been recognized since the early classic period but it was only in the latter part of the eighteenth century that it was suggested that the condition was related to peripheral nerve injury by foul wound secretions. In 1884, Nicolaier produced tetanus in laboratory animals by the injection of garden soil. Although several types of bacteria were found at the site of the soil injection, a slender bacillus occurred with such regularity that he considered it the causal agent. All efforts to cul-



tivate the bacillus failed until Kitasato applied anaerobic methods and obtained the organism in pure culture. Behring and Kitasato (1890) then demonstrated that filtrates of the cultures contained toxin, that the toxin produced characteristic tetanus in experimental animals, that repeated sublethal doses in rabbits resulted in immunity to the toxin and that the blood of the immunized animal induced passive immunity in other animals.

#### TETANUS BACILLI IN WOUNDS

The tetanus bacillus is widely distributed in the soil. In England, Fildes (1925) found it in 33 out of 70 samples of soils both from cultivated and waste land and from 34 of 200 samples of normal horse feces. It has been found less frequently in the feces of other domestic animals and man. Tulloch (1919) recovered it from 5 of 31, and Fildes (1925) from 2 of 200 samples of normal human feces. Wound tissues contaminated with soil are therefore frequently exposed to tetanus infection. As in the case of gas gangrene, tetanus bacilli are frequently present in wounds which do not develop tetanus infections. Tulloch (1919), for example, isolated *C. tetani* from 19 of 100 wounds in which there was no evidence of tetanus at the time or at a later period. On the other hand, there are a fair number of instances on record of tetanus developing weeks or months after a wound has healed.

*C. tetani* can be divided into at least ten types on the basis of specific flagellar antigens. All strains possess a common O antigen and some six types show a secondary O antigen. All types, however, produce a common toxin neutralized by one antitoxin.

#### CONDITIONS PROMOTING TETANUS INFECTION

*C. tetani* requires more rigid anaerobic conditions for cultivation in vitro than most of the gas gangrene species. In general, how-

ever, similar conditions in wounds favor the development of tetanus or gas gangrene. Fildes (1929) has stressed the effect of small amounts of calcium salts in lowering the oxidation-reduction potential of tissue to a level which will permit the germination of tetanus spores. Once growth has started and toxin formation is established, the action of toxin is probably the most important factor in preparing the ground for further proliferation of organisms. But, in sharp contrast to gas gangrene bacilli, *C. tetani* is not an invasive organism. The infection remains strictly localized in the area of the wound and frequently the volume of infected tissue is very small even in a fatal case. The disease is therefore almost entirely a toxemia.

Tetanus is not strictly limited to wound infections but occurs occasionally in association with burns and other injuries. In various parts of the world tetanus neonatorum, tetanus infection of the umbilical stump, is common. In the days of more primitive obstetrics, puerperal infections with *C. tetani* occurred with considerable frequency. Tetanus has also been associated with various surgical procedures. In the case of intestinal operations it is supposed that the infection arises from spilled intestinal contents. In other instances suture materials have been incriminated and cases of viable tetanus spores in cat gut have been recorded.

#### TOXIN

It has long been recognized that culture filtrates of *C. tetani* contain haemotoxin and lethal neurotoxin. Toxin is ordinarily produced in veal infusion peptone broth or similar media. Mueller and associates (1942) have defined the requirements for growth and toxin production in semi-synthetic media. In a medium consisting of casein hydrolysate, tryptophane, cysteine, phosphates, traces of Mn, Zn, Ca and Fe salts, together with the proper growth factors, good yields of toxin were obtained

provided the concentration of iron in the medium was held within precise limits.

The toxin is unstable in culture filtrates. It is destroyed by 5 minutes' heating to 65° C. and by exposure to acid or alkali and is readily inactivated by proteolytic enzymes. It may be precipitated from solution with ammonium sulfate and dried over sulfuric acid. Dry toxin, preserved over phosphorus pentoxide, is stable for long periods. Pickett et al. (1945) have obtained the toxin in approximately pure form by fractional precipitation with cadmium chloride; it was crystallised by Pillemer et al. in 1946. The purified toxin contains some 6,400,000 lethal mouse doses per mg. It is second only to *C. botulinum* type-A crystalline toxin in toxic activity.

Animals which succumb to tetanus intoxication show no gross or microscopic lesions. The classic works of Marie and Morax (1902) and Meyer and Ransom (1903) indicated that tetanus toxin is taken up by the motor nerve endings and reaches the anterior horn cells of the central nervous system by way of the axis cylinders. The anterior horn cells are then stimulated to produce muscular contraction. A controversy has grown up in recent years concerning the manner in which the toxin reaches the anterior horn cells and also over the question of whether involvement of the central nervous system is essential for the production of local tetanus (Abel et al., 1938; Friedemann et al., 1941).

Marie (1897) demonstrated that when a lethal dose of tetanus toxin was injected into the forepaw of a rabbit and the second cervical nerve on that side was cut, generalized tetanus failed to develop. This initial experiment led to the theory that tetanus toxin is absorbed by the motor nerves or neuromuscular junctions and transported along the axis cylinder to the principal site of action, the anterior horn cells of the cord. Friedemann et al. (1939) found that tetanus toxin injected intravenously in a guinea pig is neutralized by antitoxin injected intrave-

nously. However, a much larger dose of antitoxin is required to prevent fatal toxemia if the same amount of toxin is injected intramuscularly. It appears therefore that toxin injected intramuscularly reaches the central nervous system by some route other than the blood stream. On the other hand, Abel et al. (1935) discounted the nerve transport hypothesis. They found no anatomical evidence of the possibility of nerve transport and claimed that the toxin is carried by the blood. Sublethal doses of toxin injected at several points in a single muscle produce a local tetanus, without any generalized symptoms. Toxin injected intravenously produces both local rigidity of muscles and the reflex excitability of muscles characteristic of generalized tetanus while the application of toxin to the cord in the region of the anterior horn produces only reflex excitability. Harvey (1939) suggests that the action of toxin is associated with an increase in acetylcholine at the nerve end organs.

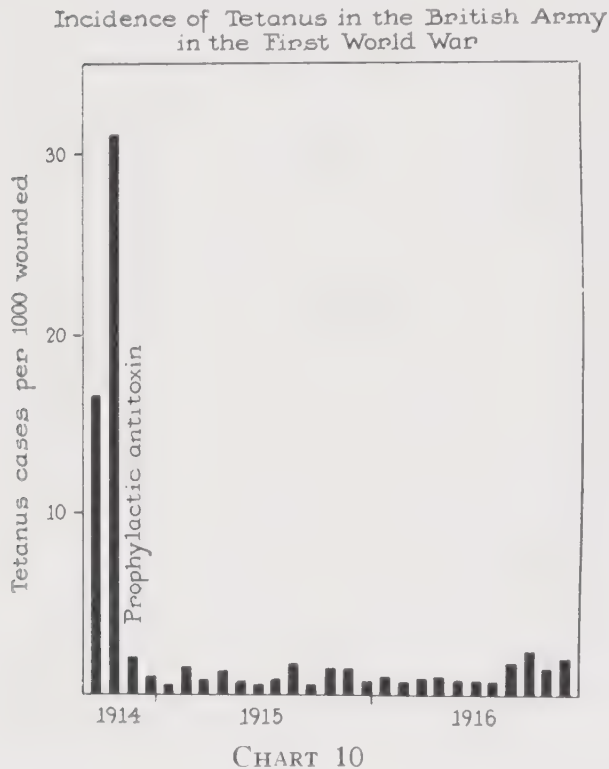
#### PROPHYLAXIS AND TREATMENT

Antitoxin is now generally prepared by the immunization of horses with toxoid, followed by toxin. The procedures in purification and standardization of antitoxin are similar to those used with other antitoxins. The American unit of antitoxin is defined as 10 times the least amount of serum necessary to save the life of a 350-gram guinea pig for 96 hours against the standard test dose of toxin. The standard dose of toxin is 100 M.L.D. of a standard toxin preserved at the National Institute of Health, Washington. Other countries have, however, adopted other standards. One German unit is equal to 66 American or 3,750 French units. The Permanent Committee on Standardization of the League of Nations (Praisnitz, 1929) has suggested an International Standard Unit equivalent to one half an American unit and based on a standard



antitoxin kept at the Serum Institute in Copenhagen.

The principle use of antitoxin is the prophylactic administration to wounded patients. The value of such treatment is clearly demonstrated by Bruce's (1920) analysis of the experience in the British Army during World War I (Chart 10). The



incidence of tetanus was very high during the first months of the war, but fell to a low level and remained low for the duration of the war following the introduction of antitoxin treatment of all the wounded.

Although it has long been considered that antitoxin is of little value once tetanus is well established, Yodh (1932) and others obtained satisfactory therapeutic results with very large doses of antitoxin, especially if given simultaneously by several routes.

In 1924, Descombey produced tetanus toxoid by the usual formalin detoxification of toxin, and it was soon found that two or three properly spaced doses of toxoid produced in a few months antitoxin blood

levels comparable with those obtained by prophylactic injection of antitoxin. Equally significant is the finding of d'Antona and Valensin (1937) that actively immunized guinea pigs, possessing 0.012 to 0.05 American units of circulating antitoxin, withstood 200 M.L.D. of toxin, whereas passively immunized animals with 5 to 10 times more circulating antitoxin failed to withstand a similar dose of toxin. Active immunization was widely practiced during World War II. The American practice was to give three doses of toxoid at intervals of three weeks, followed by a fourth dose one year later or before military operations (Long, 1943). The wounded were given a further dose immediately after the injury. Details of procedure varied somewhat in other armies. The British actively immunized in a similar manner and gave the wounded a prophylactic dose of antitoxin. Tetanus was an extremely rare disease during World War II.

## BOTULISM

### HISTORY

During the eighteenth century, the term botulism was applied in South Germany to a complex of neuromuscular symptoms supposed to be associated with the consumption of spoiled sausages. In 1896 van Ermengem cultivated from ham incriminated in an outbreak of botulism in Belgium an anaerobic bacillus which he described under the name *Bacillus botulinum*. He demonstrated that the culture produced a powerful toxin which gave rise to the same symptoms as the original ham. It was later shown that treatment of goats with sublethal doses of toxin resulted in the formation of antitoxin.

### SPECIES AND TYPES

Two sub-species of *C. botulinum* have been recognized by Bengston (1924): *C. parabotulinum* which digests egg albumin

and *C. botulinum* which does not. However, since there is no relationship between albumin digestion and toxin production, this difference is of doubtful significance. It has long been known, on the other hand, that different strains produce specific toxins. Burke (1919) distinguished two types, A and B, each producing a toxin neutralized only by homologous antitoxin. More recently types C, D and E have been differentiated. The reservoir of all these organisms is the soil and their spores occur on a wide variety of vegetation, vegetables and fruits.

#### SOURCES OF HUMAN CASES

The disease is a toxemia and not an infection, as there is no evidence that the organisms multiply in the human body. Disease results from the ingestion of foods in which the organisms have grown and produced the toxin. Most European cases have been traced to smoked, salted or spiced meats and most American to canned vegetables or fruits. In all instances, the foods have been preserved in some form, kept for a time, and eaten without further cooking. Cases have not been associated with fresh foods, cooked or raw. During the last thirty years, in the many outbreaks traced to canned foods, the incriminated products have always been domestically and not commercially canned. Inadequate sterilization is the invariable cause.

The disease is not common. From 1896 to 1925 there were 146 outbreaks in the United States involving 503 persons with 337 deaths. Other parts of the world show a more or less similar incidence. Human cases are mostly due to types A or B, with a very few outbreaks due to type E. Types C and D have not been associated with human cases. Type C is responsible for epidemics in domestic fowls and wild ducks. Types C and D have frequently been associated with forage poisoning in cattle, horses and sheep.

#### TOXIN

Stevenson et al. (1947) found that a type A strain grown for 4 days under anaerobic conditions in a tryptic digest of casein supplemented with glucose and corn-steep liquor produced from 1 million to 3 million lethal-mouse doses per milliliter of culture. Growth reached a peak in less than 24 hours and was followed by gradual autolysis of the bacteria. Although toxin could be demonstrated during the early growth period, its concentration reached a maximum only after complete autolysis of the bacteria. The toxin of type A can be precipitated from autolyzed cultures at pH 3.5 and dried in a stable form. It has been crystallized and characterized as a protein containing 14 per cent of nitrogen, 0.1 per cent phosphorus and some 14 amino acids (Lamanna et al., 1946; Abrams et al., 1946). The M.L.D. is about 0.00005  $\gamma$  for the white mouse (i.e. one milligram contains about 20 million mouse lethal doses). This is the most toxic substance known. The lethal dose for man is not known, but it may be noted that toxicity of type A toxin is approximately the same, per gram of body weight, for several species of mammals. Type B is much more toxic for guinea pigs than for mice.

It is of special interest that botulinus toxin, unlike tetanus and diphtheria toxins, is relatively resistant to the action of pepsin and trypsin, and is therefore effective when administered by mouth. Botulinus toxin is a neurotoxin. According to Bishop and Bronfenbrenner (1936), it acts specifically on the myoneural junctions and has a "curarelike" action. Death is due to respiratory paralysis.

#### SYMPTOMS AND PATHOLOGY

The incubation period in man following the ingestion of food containing toxin varies with the amount of toxin ingested, from less than 24 hours to several days. The signs and symptoms are vomiting, constipation, thirst, oculomotor, pharyngeal and re-



spiratory paralysis. Consciousness is maintained until near death, which is preceded by delirium and coma. The temperature is generally subnormal. Death may occur in less than 20 hours from the onset or may be delayed for more than a week. In surviving cases partial paralysis may persist for six to eight months. As in the case of tetanus, very little is to be found at necropsy: kidneys, liver and meninges usually show slight congestion, and points of thrombosis may be found.

#### TREATMENT

**Toxoids** of good antigenic value have been prepared by detoxifying the toxin with

formalin in the usual manner. The disease is not sufficiently common to justify their use except in laboratory workers.

**Antitoxins** have not given as good therapeutic results as might be expected from neutralization tests in vitro. It is of course essential to use antitoxin homologous with the toxin, but, since identification of the type concerned is frequently time consuming, it is customary to use polyvalent A and B antitoxin since these types are most commonly encountered. It is probable that some of the disappointing results have been due to the use of inadequate amounts of antitoxin.

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# 16

## The Enteric Bacteria

### INTRODUCTION

The enteric group of bacteria (*Enterobacteriaceae*) includes a large number of Gram-negative, nonsporulating rods whose natural habitat in most instances is the gastro-intestinal tract of man and other animals. Some are definitely pathogenic for man and are the etiologic agents of various types of gastro-intestinal diseases such as typhoid and other enteric fevers, gastro-enteritis (*Salmonella*) or dysentery (*Shigella*). Others (e.g. the *Escherichia*) appear to lead a saprophytic existence in the intestinal tract but may cause pathologic processes in other parts of the body such as the genito-urinary and respiratory systems. The *Aerobacter* group occurs most commonly in nature in soil and on grain. There are no simple differential criteria for these organisms, and classification is based on morphologic characteristics, biochemical reactions, antigenic properties and ecologic considerations. Even when all these criteria are invoked, some organisms fail to exhibit all the characteristics of a single group, appearing to occupy an "intermediate" position between two of the main groupings.

As a general rule, the enteric bacilli grow readily on the ordinary media. They are aerobes or facultative anaerobes, and characteristically ferment a wide range of carbohydrates. Many are actively motile and at least one group commonly possesses

easily demonstrable capsules. Their antigenic structure forms a complex mosaic which often results in serologic interrelationships between different genera and species.

The enteric bacteria will be considered under the following groups:

**The coliform group** is characterized by the prompt fermentation of lactose usually with the production of acid and gas. *Escherichia coli* is a normal, apparently harmless inhabitant of the intestinal canal but frequently causes infections of the urinary tract and other organs. The closely related organism *Aerobacter aerogenes* is most frequently found in soil and on grain. Certain other closely related bacilli are usually classified with these organisms. The paracolon bacilli ferment lactose slowly, frequently only after several days of incubation. Their habitat is the intestinal tract of man and animals where, for the most part, they have no significance as causative agents of gastro-intestinal disease. *Klebsiella pneumoniae* which is encapsulated and nonmotile is a saprophyte of the intestinal and upper respiratory tracts, which, however, may cause inflammatory lesions in the lower respiratory tract and elsewhere.

**The Salmonella** include the causative agents of typhoid fever, paratyphoid and other enteric fevers; gastro-enteritis; various types of septicemic infections; and certain diseases of the lower animals as well.

TABLE 35. BIOCHEMICAL REACTIONS OF ENTERIC BACTERIA

ORGANISM	MO- TILITY	FERMENTATION REACTIONS													INDOL	HY- DRO- GEN SUL- FIDE	GELA- TIN LIQUE- FAC- TION	RUSSELL'S DOUBLE SUGAR		CIT- RATE UTILI- ZATION	ACE- TYL- METHYL CARBI- NOL VP	METH- YL RED	TRI- METHYL AMINE OXIDE REDUC- TION
		GLU- COSE	LAC- TOSE	SU- CROSE	MAN- NITOL	SALI- CIN	RHAM- NOSE	DUL- CITOL	INOSI- TOL	SORBI- TOL	ARAB- NOSE	XY- LOSE	SLANT	BUTT									
<i>E. coli</i> .....	+	AG	AG	V	AG	V	AG	V	-	AG	AG	AG	+	-	A	AG	-	+	+	-			
<i>A. aerogenes</i> .....	V	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-	A	AG	+	+	-				
<i>Paracolon</i> .....	V	AG	(V)						AG	AG	AG	AG			Alk.	AG	V	V	V				
<i>Kl. pneumoniae</i> .....	-	AG	V	V	V		-	-	V	A	-	V	V	+	Alk.	A							
<i>S. typhosa</i> .....	+	A	-	-	AG	-	AG	AG	-	AG	AG	-	-	-	Alk.	AG							
<i>S. paratyphi</i> .....	+	AG	-	-	AG	-	AG	AG	V	AG	AG	AG	+	+	Alk.	AG							
<i>S. schottmuelleri</i> .....	+	AG	-	-	AG	-	AG	AG	-	AG	AG	AG	-	-	Alk.	AG							
<i>S. hirschfeldii</i> .....	+	AG	-	-	AG	-	AG	AG	V	AG	AG	V	+	+	Alk.	AG							
<i>S. typhimurium</i> .....	+	AG	-	-	AG	-	AG	V	-	AG	-	AG	-	-	Alk.	AG							
<i>S. choleraesuis</i> .....	+	AG	-	-	AG	-	AG	AG	-	AG	AG	AG	-	-	Alk.	AG							
<i>S. enteritidis</i> .....	+	AG	-	-	AG	-	AG	AG	-	AG	AG	AG	-	-	Alk.	A							
<i>S. gallinarum</i> .....	-	A	-	-	A	-	-	A	-	-	A	A	-	-	Alk.	A							
<i>Sh. dysenteriae</i> .....	-	A	-	-	-	-	A	-	-	V	V	-	-	-	Alk.	A							
<i>Sh. ambigua</i> .....	-	A	-	-	-	-	A	-	-	V	A	V	+	-	Alk.	A							
<i>Para-shiga group</i> .....	-	A	-	-	-	-	V	(V)	-	V	A	-	V	-	Alk.	A							
<i>Sh. paradyenteriae</i> .....	-	A	-	V	A*	-	V	V	V	V	V	-	V	-	Alk.	A							
Flexner subgroup.....	-	A	-	-	A	-	-	V	V	(V)	A	(V)	V	-	Alk.	A							
Boyd subgroup.....	-	A	-	-	A	-	A	V	V	V	A	A	V	-	Alk.	A							
<i>Sh. alkalescens</i> .....	-	A	-	-	A	V	A	V	-	V	A	A	+	-	Alk.	A							
<i>Sh. sonnei</i> .....	-	A	(A)	(A)	A	-	A	-	-	-	A	V	-	-	Alk.	A							
<i>Sh. dyspar</i> .....	-	A	(A)	(A)	A	-	A	V	-	A	A	A	+	-	Alk.	A							
<i>Proteus vulgaris</i> .....	+	AG	-	AG	A	-	A	-	-	-	-	-	-	+	Alk.	AG	V	-	-				
<i>Proteus morgani</i> .....	+	AG	-	AG	-	AG	-	-	-	-	-	-	-	-	Alk.	AG	-	+	+				
<i>Ps. aeruginosa</i> .....	+	A	-	-	-	-	-	-	-	-	-	-	-	-	Alk.	AG	-	+	+				
<i>Alcaligenes fecalis</i> .....	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Alk.	-							

A = Acid. AG = Acid and gas. Alk. = Alkaline. + = Positive. - = Negative. V = Variable. () = Delayed.  
 \* = A few strains fail to ferment mannitol.



Characteristically they fail to ferment lactose. On account of its importance, the salmonella group is considered independently in Chapter 17.

The *Shigella* include the causative agents of bacillary dysentery in man. In contrast to those of other groups, these organisms are nonmotile and, with one important exception, fail to ferment lactose. On account of its importance, the shigella group will be considered independently in Chapter 18.

The *Proteus* group is comprised of organisms widely distributed in nature. Although they are frequently found in the intestinal tract of man, their causal relationship to enteric disease is, with one exception, doubtful. Elsewhere in the body they may cause primary or secondary infections especially in the genito-urinary tract. They are actively motile, decompose urea and fail to ferment lactose.

For the sake of convenience, the *Pseudomonas* bacilli and *Alkaligenes fecalis* will be considered at the end of the present chapter.

The biochemical reactions of the enteric bacteria are presented in Table 35.

### THE COLIFORM BACILLI \*

The coliform group includes the following Gram-negative bacilli: *Escherichia coli*, *Aerobacter aerogenes*, the paracolon bacilli and *Klebsiella pneumoniae*. The first two are often referred to as the colon-aerogenes group. *E. coli* is typically a normal inhabitant of the intestinal tract of man and animals. *A. aerogenes* is found most frequently on grains and plants but also occurs in the feces of man and animals. Because of its predominantly intestinal origin, *E. coli* is used as an indication of pollution of water with fecal material, whereas the presence in water of other bacteria which, like *A. aerogenes*, have other natural habi-

tats does not necessarily indicate fecal pollution.

Organisms of the colon-aerogenes group cause infections in man which are primarily of a localized nature often involving the genito-urinary tract or organs having an anatomic relationship to the intestinal tract, e.g., gallbladder, peritoneum and appendix.

### ESCHERICHIA COLI

*E. coli* (Synonym: *Bacterium coli*) was isolated from feces by Escherich in 1885. It is found universally in the intestinal tract of man and animals, and, being the predominant organism in the colon, is commonly referred to as the "colon bacillus."

*E. coli* is a Gram-negative bacillus which commonly occurs as short rods, from 2 to 3  $\mu$  long and about 0.6  $\mu$  in breadth, and which may form chains. Very long, filamentous forms are occasionally seen. Most strains are motile. *E. coli* does not produce spores and, like other members of the group, usually does not possess a readily demonstrable capsule; however, capsules are produced under special conditions such as growth at low temperatures (Morgan and Beckwith, 1939).

*E. coli* is facultatively anaerobic and grows on all ordinary laboratory media. The optimum incubation temperature is about 37° C. On beef extract agar, it usually forms circular, convex, smooth, colorless colonies with regular edges, but some colonies, which probably represent rough dissociants, may have an irregular surface and edge. By transmitted light, the growth has a granular appearance. On blood agar, some strains produce hemolysis. Growth in broth appears as a uniform turbidity with some sediment. Cultures have a characteristically fetid odor.

*E. coli* ferments a variety of carbohydrates including dextrose, lactose, maltose, mannitol and xylose, but not dextrin or starch, with the production of acid and gas. Sucrose, salicin and raffinose are attacked

\* This section was prepared by Herbert R. Morgan, M.D.

by some strains, but not by others. The action on sucrose is the basis for the classic differentiation of colon bacilli into two fermentative varieties: *E. coli* var. *communior*, which ferments sucrose, and *E. coli* var. *communis*, which does not. Another fermentative variety, *E. coli* var. *acidilactici*, is characterized by its failure to ferment both sucrose and salicin. Occasionally there are isolated strains of *E. coli* which fail to ferment lactose with the characteristic rapidity, although these same strains readily produce variants which immediately ferment the sugar. Because of this property, these strains have been designated *E. coli mutabile*. Colon bacilli form indol and do not liquefy gelatin;  $H_2S$  is not usually produced.

Colon bacilli are usually killed at a temperature of  $60^\circ C$ . for 30 minutes. They are more susceptible than the salmonella to the inhibitory action of such compounds as brilliant green dye and sodium desoxycholate. The composition of certain differential media which are designed for the isolation of Salmonella from the feces, is based upon these differences in susceptibility.

*E. coli* is serologically heterogeneous, and the antigenic pattern of the various species has been studied in some detail (Stuart et al., 1940). Some strains have somatic antigens which are similar in antigenic structure to those which occur in the salmonella organisms.

*E. coli* undergoes dissociation to give rough and smooth colonial types. The smooth, round, translucent colonies of the S form contrast with the irregular colonies of the R form which have a dull surface and opaque character. Mucoid forms occur and usually appear when cultures are grown at low temperatures.

Whereas *E. coli* is a harmless and perhaps useful inhabitant of the intestines of man and animals, it may, under certain conditions, assume the role of a pathogen, especially in the invasion of organs anatomically related to the intestinal tract such as

the appendix, gallbladder, peritoneal cavity, kidneys and bladder. In appendicitis and peritonitis the colon bacillus commonly occurs in the tissues along with a variety of other organisms. It is one of the most common invaders of the peritoneum following perforation of some part of the intestinal tract. *E. coli* appears to be the predominant organism in many cases of the suppurative form of cholecystitis. Acute infections of the urinary tract including pyelitis, pyelonephritis and cystitis are caused most frequently by *E. coli* (Parr, 1939). Along with the other organisms that may occur on the skin, *E. coli* is also found in wound infections but much less frequently than streptococci or staphylococci, except when the wound has been contaminated with urine or feces. Colon bacilli may gain access to the blood stream, particularly in infants, in the agonal stages of diseases and immediately after death.

*E. coli* is susceptible to the therapeutic action of the sulfonamide drugs but not of penicillin. The sulfonamides are useful in the treatment of urinary-tract infections and may be used prophylactically to prevent infections due to the contamination of the peritoneal cavity with fecal material as a result of injury or surgical operations. Streptomycin is also useful in therapy.

#### AEROBACTER AEROGENES

*A. aerogenes* (Synonym: *Bacterium aerogenes*), also described by Escherich, occurs less frequently in the intestinal tract than *E. coli* and is found more often on grains and plants (Griffin and Stuart, 1940). The difference in the usual habitat of these two closely related organisms has led to a detailed study of their specific differentiation because of the practical importance of the use of *E. coli* as an indicator of the fecal pollution of water.

*A. aerogenes* is often shorter than *E. coli* and more commonly exhibits easily demonstrable capsules. It is facultatively anaero-



bic, and may be motile or nonmotile. Growth on agar is often of a mucoid character with convex, smooth colonies. It grows more profusely at temperatures below 37° C. with an optimum near 30° C. Growth in broth produces a pellicle and a more viscous deposit.

*A. aerogenes* differs from *E. coli* in producing less acid but more gas during fermentation and in frequently fermenting dextrin and starch. Several biochemical reactions are of special importance for the differentiation of these two organisms (Tables 35 and 36). *A. aerogenes* does not produce

other different combinations. These organisms are known as intermediates. Their occurrence suggests that, in spite of the classic differences described, coliform organisms are best considered as a closely related and intergrading group in which variant forms occur as a result of loss or acquisition of certain characteristics. *A. aerogenes* may cause, in man, urinary tract infections which respond to the same therapy as infections due to *E. coli*. Differential reactions for coliform organisms are presented in Table 36.

TABLE 36. DIFFERENTIAL REACTIONS FOR COLIFORM ORGANISMS

ORGANISMS	INDOL	M.R.	V.P.	CITRATE MEDIUM
<i>E. coli</i> .....	+	+	-	-
<i>A. aerogenes</i> .....	-	-	+	+
<i>Kl. pneumoniae</i> .....	V	V	V	V

indol. In dextrose broth, it produces less acid than *E. coli* as shown by the methyl red test, and produces acetylmethylcarbinol which is responsible for a positive Voges-Proskauer reaction. *A. aerogenes* is able to utilize sodium citrate as the sole source of carbon in a synthetic medium while *E. coli* produces very little if any growth in such a medium. These four differential criteria (the production of indol, methyl red and Voges-Proskauer tests and citrate utilization) are sometimes referred to as the "I M Vi C" group.\* The ability to produce gas from glucose when incubated at 46° C., called the Eijkman reaction, has also been used as a differential test since *E. coli* grows freely and produces gas at this temperature while *A. aerogenes* does not.

When coliform bacilli are classified on the basis of the "I M Vi C" tests, some organisms give reactions which are intermediate between those of *E. coli* and *A. aerogenes*, e.g., Indol+, MR+, VP—, Citrate+, and

\* The group reactions for *E. coli* are Indol+, MR+, VP—, Citrate— and for *A. aerogenes* Indol—, MR—, VP+, and Citrate+.

PARACOLON BACILLI

The organisms grouped together under the term "paracolon" bacilli do not ferment lactose, or only attack it after prolonged incubation, and therefore lie outside the classic coliform group in which immediate fermentation of lactose is the basic differentiating characteristic. They are not salmonella because they may slowly ferment lactose, sucrose or salicin; they lack the characteristic antigenic patterns of these organisms and are not primarily pathogenic like them (Stuart et al., 1943). The paracolon bacteria then appear to lie between the normal coliform bacteria and the salmonella and provide another instance of the intergrading relationships in the enteric group.

The presence of paracolon bacilli in the intestine complicates the examination of fecal specimens for organisms of pathological significance since they possess various combinations of the several characteristic reactions on which the differential isolation technics for pathogens and non-

pathogens are based. The organisms within the group are markedly heterogeneous in their biochemical and antigenic properties.

Paracolon bacilli seem to occur in feces more commonly during outbreaks of gastroenteritis (Stuart et al., 1943). They also are isolated from the feces of normal persons, especially in tropical areas, so that they probably are not primary agents in the etiology of disease. However, since some possess the ability to invade tissues of the intestinal or urinary tracts under certain conditions their role is probably that of secondary invaders. These bacteria and others in the coliform group may contain one or more of the somatic antigens found in the salmonella but never the complete antigenic complement characteristic of any *Salmonella* species (Wheeler et al., 1943).

The group is markedly heterogeneous in its biochemical and antigenic properties.

#### KLEBSIELLA PNEUMONIAE AND THE FRIEDLÄNDER GROUP\*

The organisms of the Friedländer group (Synonyms: pneumo bacillus, *Bacterium friedländeri*, *Bacillus mucosus capsulatus*, Friedländer's bacillus) are short, nonmotile, nonsporing, Gram-negative bacilli which characteristically possess large capsules. They produce large, mucoid colonies on agar media and ferment a number of carbohydrates with the production of acid and gas. They are found in the nose, mouth and intestinal tract of normal persons, in the lungs of patients with pneumonia and other respiratory diseases, and in suppurative infections of other parts of the body.

*Klebsiella pneumoniae* is the most important member of the group. It was discovered in 1883 by Friedländer in the lungs of patients dying with pneumonia and is now known to cause a small proportion of the bacterial pneumonias.

\* This section was prepared by Herbert R. Morgan, M.D.

#### MORPHOLOGY

In infected tissues, *Kl. pneumoniae* usually occurs as a rod from 2 to 5  $\mu$  long and 0.5  $\mu$  thick, either singly or in pairs. In cultures, it shows pleomorphism with curved rods, long filaments and other forms. It occurs naturally in the mucoid phase with a capsule which is usually visible even in an ordinary Gram stain and which is particularly striking when the organisms are grown on media rich in carbohydrate. A profuse, mucoid growth of a tenacious character is produced on solid media. It is luxuriant and viscous in broth. After repeated subculture, *Kl. pneumoniae* tends to lose its mucoid character and dissociates to give smooth colonies made up of organisms which do not produce the characteristic large capsules. Reversion to the mucoid form may occur on further subculture.

#### BIOLOGIC CHARACTERISTICS

*Kl. pneumoniae* is facultatively anaerobic and grows best at 37° C. with a range from 15 to 40° C. Growth is luxuriant on ordinary nutrient media. The organisms are killed by moist heat at 55° C. in 30 minutes.

The biochemical reactions are highly variable from strain to strain. These variations may occur within a single serologic type and increase tremendously the difficulty of classification. *Kl. pneumoniae* usually ferments glucose, maltose, lactose, sucrose, mannitol and salicin with the production of acid; some strains fail to produce gas. Indol production is variable. The organism is usually MR+, VP-, and does not produce H<sub>2</sub>S.

The morphologic and biochemical properties of *Kl. pneumoniae* show that it is closely related to *E. coli* from which it is chiefly distinguished by its respiratory habitat, its etiologic role in certain cases of pneumonia in man and its characteristic possession of an easily visible capsule. The differentiation from *A. aerogenes* is much



more difficult. It becomes indistinguishable from other coliform organisms when it undergoes dissociation from the mucoid to the smooth form and thereby loses its large capsule (Osterman and Rettger, 1941).

The antigenic structure of *Kl. pneumoniae* has been studied by Julianelle (1926) who found that the capsule contains a type-specific polysaccharide. He described 3 serologic types, A, B, C, and a fourth heterogeneous group X. The specific polysaccharide of the type B organism shows an immunologic relationship to that of the type 2 pneumococcus. A nucleoprotein which is common to all types is found in the body of the bacterial cell.

#### PATHOGENICITY

*Kl. pneumoniae* is found in the respiratory tract of from about 1 to 5 per cent of normal individuals (Bloomfield, 1921) and frequently occurs as a secondary invader in the lungs of patients with bronchiectasis, influenza and tuberculosis. It is the primary cause of pneumonia in from 1 to 3 per cent of all bacterial pneumonias (Hyde and Hyde, 1943). It has been isolated from patients with pleurisy, appendicitis, cystitis and pyelonephritis and from the feces of about 5 per cent of normal individuals (Baehr et al., 1937). There is some evidence that it may play an etiologic role in diarrhea in infants (Walcher, 1946). It occurs frequently in abdominal infections though its presence is often overlooked in the bacteriologic studies.

In animals, *Kl. pneumoniae* has been isolated from spontaneous respiratory diseases of mice and in a metritis occurring in mares. Types A and B are highly pathogenic for mice by intraperitoneal injection while guinea pigs and rabbits show a higher degree of resistance. Type C appears to be relatively avirulent for animals.

In pneumonic infections in man, *Kl. pneumoniae* is notable for its destructive action on the tissues and for the production of

small areas of abscess formation throughout the lung. Julianelle (1941) found type A in 64 per cent, type B in 14 per cent, type C in 7 per cent, and group X types in 15 per cent of a series of cases of pneumonia. The mortality of untreated cases is high and may reach 90 per cent in those with bacteremia (Solomon, 1947). Chronic Friedländer bacillus infections of the lung may follow the acute pneumonia.

Sulfadiazine has proven of value in therapy, and more recently streptomycin has produced dramatic results even in patients with bacteremia (Solomon, 1947).

#### OTHER ORGANISMS OF THE FRIEDLÄNDER GROUP

Two other encapsulated, mucoid, Gram-negative bacilli resembling *Kl. pneumoniae* and occurring characteristically in the mucoid phase have been isolated from disease conditions of the upper respiratory tract of man. Frisch in 1882 isolated an encapsulated organism from the granulomatous nasal lesions of patients with rhinoscleroma, and a similar organism was isolated by Loewenberg in 1894 from the nasal secretions of individuals with ozena, a fetid, catarrhal condition of the nose. The rhinoscleroma organism is antigenically identical with type C. Friedländer's bacillus, but *Kl. ozenae* may be easily differentiated from *Kl. pneumoniae* and *Kl. rhinoscleromatis* by serologic tests.

The salmonella and shigella groups should logically be discussed at this time, as they are closely related to the coliform bacilli; as mentioned before, however, they will be considered in Chapters 17 and 18, respectively.

#### THE PROTEUS GROUP\*

##### INTRODUCTION

The proteus group consists of pleomorphic Gram-negative bacilli which do not

\* This section was prepared by Herbert R. Morgan, M.D.

ferment lactose and which are characterized by their active motility and spreading growth on solid media. They are commonly found in sewage and manure and occur in normal human stools. They also play a role in pathologic conditions of the genitourinary and gastro-intestinal tracts of man. The two commonest pathogenic species are *Proteus vulgaris* and *Proteus morgani*; other strains are of medical importance because of their antigenic relationships to certain rickettsiae.

#### PROTEUS VULGARIS

*Proteus vulgaris* is an actively motile, Gram-negative rod-shaped organism which is subject to great variation in size and shape. The more typical forms in agar cultures average 1 to 3  $\mu$  long and 0.4-0.6  $\mu$  wide, but short coccobacillary forms are also seen. The rods occur singly, in pairs and frequently in long chains. Young cultures which show swarming are particularly pleomorphic and may include long and filamentous forms.

The organism is a facultative anaerobe with an optimum growth range from 34° to 37° C. but able to grow well at 20° C. on solid moist media. It spreads rapidly from the initial colonies over the entire surface by a process called "swarming" which is due in part to the very active motility of the bacilli. Swarming may be prevented by increasing the agar content of the media to 6 per cent. In broth the organism gives a moderate uniform turbidity with some deposit.

*Proteus vulgaris* produces acid and gas in glucose, sucrose and galactose. Some strains ferment maltose. The maltose-fermenting strains which form indol are VP negative and usually fail to grow on citrate agar, while those which do not ferment maltose are indol negative, usually VP positive and usually grow on citrate agar. It exhibits active proteolytic action and liquefies gelatin,

digests casein and decomposes urea. It produces  $H_2S$ ,  $NH_3$  and reduces nitrates.

The antigenic structure of *Proteus vulgaris* has received considerable attention because of the use of certain strains designated by the letter X in the Weil-Felix reaction for the diagnosis of typhus fever and of other rickettsial diseases. The group as a whole is antigenically heterogeneous with differences in both H and O antigens (Rustigian and Stuart, 1945). On the basis of their serologic relationships to certain rickettsiae, the O antigens of the X strains have been divided into three types: OX2, OX19 and OXK. Castaneda (1934) reported the isolation from *Proteus* OX19 of a soluble, specific polysaccharide which seems to be also present in *Rickettsia prowazeki* and to be responsible for the serologic reaction utilized in the Weil-Felix test in epidemic typhus fever.

Organisms of the *Proteus* group are widely distributed in nature. They form an important part of the flora of decomposing animal and vegetable matter in manure and sewage. The organisms occur in the feces of man and animals; they occur in large numbers only when some abnormal condition exists. *P. vulgaris* tends to become more prominent when stool specimens are incubated in the enrichment media, tetrathionate broth or selenite F used for suppressing *E. coli* in the isolation of enteric pathogens. In addition to its saprophytic existence, *P. vulgaris* multiplies in the animal body under certain conditions and may be isolated in pure or mixed cultures in urinary tract infections, from abscesses or wounds, and in peritonitis. It causes from 6 to 13 per cent of human urinary tract infections (Pierson and Honke, 1941). In some instances, it has been isolated from cases of gastro-enteritis where it appeared to play an etiologic role (Cooper et al., 1941). Isolation of the X strains from the urine and feces of patients with typhus fever led to the study of the possible (now discounted) etiologic relationship to typhus and to the



discovery and use of the Weil-Felix reaction.

The intraperitoneal inoculation of *Proteus vulgaris* into mice, rats, guinea pigs or rabbits often causes death of these animals but strains vary greatly in pathogenicity.

The sulfonamides are of limited value in the treatment of infections caused by *Proteus vulgaris*. Streptomycin has proven useful in many cases.

#### PROTEUS MORGANI

*Proteus morgani* (Morgan's bacillus) was isolated by Morgan from the stools of patients with diarrhea. Most strains show the swarming characteristics of the *Proteus* group, usually at lower incubation temperature, e.g., 20° C., but tend to lose this property on cultivation. Likewise, motility is pronounced at room temperature, but it decreases or it is lost at 37° C. Although Morgan's bacillus is related closely to *P. vulgaris*, as shown by its ability to swarm and to decompose urea, it does not liquefy gelatin or produce H<sub>2</sub>S. It produces indol and ferments, for the most part, only monosaccharides.

*Proteus morgani* has been isolated on a number of occasions from outbreaks of infantile diarrhea (Neter and Farrar, 1943) where it seemed to play an etiologic role, but it has also been isolated from stools of normal persons. It may occasionally cause infections of the genito-urinary tract in man and rarely other purulent lesions. Spontaneous epidemics of an enteritis due to it have been observed in mice.

#### MISCELLANEOUS GRAM-NEGATIVE BACILLI \*

##### PSEUDOMONAS AERUGINOSA

The pseudomonas group is composed of Gram-negative, rod-shaped, motile organ-

isms, which characteristically produce a water-soluble pigment which diffuses through the medium. They occur widely in water and soil. Some species are pathogenic and the type species *Pseudomonas aeruginosa* (*Pseudomonas pyocyanea*, *Bacillus pyocyaneus*) occurs in wound and urinary tract infections in man.

Gessard in 1882 isolated *Ps. aeruginosa* from the "blue pus" found in some wound infections. This organism is closely related to about thirty other species of *Pseudomonas* which occur principally in soil, water and sewage, although some produce disease in animals and plants. *Ps. fluorescens* is one of the most common of these other species.

*Ps. aeruginosa* is a Gram-negative, motile rod measuring 1.5 to 3.0  $\mu$  by about 0.5  $\mu$ . It is not encapsulated and forms no spores. It grows readily on all ordinary culture media. On agar it forms round, smooth, moist glistening colonies which have a fluorescent yellowish-green color, although most of the pigment diffuses into the medium coloring it bluish-green. The organism is aerobic and grows best at from 30 to 37° C. It is killed at 55° C. for one hour.

It is not an active fermenter of carbohydrates and produces acid, but no gas, in glucose. It actively liquefies gelatin, produces ammonia and grows on citrate medium. *Ps. aeruginosa* does not produce indol, is methyl red and Voges-Proskauer negative; it fails to produce hydrogen sulfide and to reduce nitrates.

The bluish-green pigment produced by *Ps. aeruginosa* consists of two substances: pyocyanin, a bluish-green pigment soluble in chloroform and water, and fluorescein, which is greenish-yellow, fluorescent and soluble in water but not in chloroform. The closely related organism, *Ps. fluorescens* forms only fluorescein. These pigments are antibacterial for certain other organisms (Schoental, 1941).

*Ps. aeruginosa* is the only member of this group pathogenic for man, and its pathogenicity is of a low order. It is found occa-

\* This section was prepared by Herbert R. Morgan, M.D.

sionally in the human intestine and on the skin as well as in water and sewage. It may produce local suppurative lesions, especially skin and wound infections and otitis media. It may occur in infections of the genitourinary tract, respiratory tract, the joints and the eye. Meningitis due to it has been observed to follow a lumbar puncture or operative exposure of the meninges. In some outbreaks of a dysenterylike enteric infection, *Ps. aeruginosa* has been isolated under circumstances which suggest an etiologic role.

It produces fatal infections when injected subcutaneously or intravenously into guinea pigs or rabbits.

Streptomycin has proven of value in treating infections caused by *Ps. aeruginosa*.

#### ALCALIGENES FECALIS

*Alcaligenes fecalis* is a Gram-negative rod which is found in human feces and can be confused with *Salmonella typhosa* since it does not ferment lactose and therefore produces similar colonies on the usual differential media used for the isolation of enteric pathogens. It is readily distinguished from other Gram-negative organisms by its failure to ferment glucose as well as most other carbohydrates. It may, in rare instances, cause enteric infections in man.

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## 17

# The Salmonella

### INTRODUCTION

The salmonella are Gram-negative, non-spore forming, motile bacilli easily cultivated on ordinary media, and they characteristically fail to ferment lactose and sucrose. The different species are closely related antigenically, and these relationships are used as the main criterion in classification. All are pathogenic for man or animals or frequently both. *Salmonella typhosa*, the cause of typhoid fever is pathogenic only for man, while the other salmonella produce disease in man and animals.

### HISTORY

Before the development of modern bacteriology and the isolation of the typhoid bacillus, William Budd, in 1856, made a strikingly accurate study of typhoid fever outbreaks which led him to believe that the disease was contagious and that the infectious agent was excreted in the feces of patients. He believed that contaminated milk and water probably also played a role in the spread of the disease. In 1880, Eberth described the typhoid bacillus in tissues of patients and the organism was isolated by Gaffky in 1884. Following this, other *Salmonella* were isolated from patients with typhoidlike fevers, and it became obvious that a clinical syndrome similar to typhoid fever might be caused by a variety of closely related organisms.

Gaertner in 1888 isolated *Salmonella enteritidis* from a patient who died following the consumption of meat contaminated with

this organism, and shortly afterward Durham and de Noeble described another organism, *S. typhimurium*, isolated from patients suffering with gastro-enteritis following again the ingestion of infected meat. Further studies rapidly increased the numbers of organisms identified as having the typical properties of members of the *Salmonella* group.

### MORPHOLOGY

*Salmonella* average from about 2 to 3  $\mu$  in length and about 0.6  $\mu$  in width, but may show variation in size under different environmental conditions. Young cultures on agar may present a predominance of coccobacillary organisms, while filamentous forms are occasionally seen especially in cultures in liquid media. With the exceptions of *S. gallinarum* and *S. pullorum*, all strains are motile with peritrichal flagella. *Salmonella* do not ordinarily form capsules when grown at 37° C., but most species may give rise to mucoid colonies composed of encapsulated bacilli, especially when incubated at temperatures of 20° C. or lower. Encapsulated strains of *S. typhosa* have been described.

### CULTIVATION AND BIOCHEMICAL REACTIONS

*Salmonella* grow readily on ordinary culture media producing in 24 to 48 hours colo-

nies which average 2 to 3 mm. in diameter and which are indistinguishable from those of the coliform bacteria. The colonies may be circular with a smooth surface and an even edge or flat with an uneven surface and serrated edge. On plates incubated at 37° C. and then allowed to stand at room temperature, they may show a secondary growth of mucoid character around the original margin. *Salmonella* in the smooth phase produce a uniform turbidity in broth with a deposit which readily resuspends on shaking. They may produce heavy growth under anaerobic conditions. The temperature range of growth is from about 10° to 42° C. with an optimum at 37° C.

The biochemical reactions serve to define the salmonella as a group and also provide an aid in the differentiation of certain species (Table 35). By definition, these bacteria do not ferment lactose, sucrose or salicin while glucose, mannitol, maltose and dextrin are fermented with the production of acid and gas except in the case of *S. typhosa* and *S. pullorum* which do not form gas. Arabinose, xylose, trehalose and inositol are useful in the differentiation of certain species and varieties: e.g., *S. paratyphi* does not ferment xylose, while *S. schottmuelleri* does. The fermentation of tartrate varies with different species, while almost all attack citrate. Indol is not produced, and gelatin is not liquefied, and, since there are very few exceptions to these two metabolic activities, they serve along with the failure to ferment lactose as the most reliable biochemical differential characters.

#### EFFECTS OF PHYSICAL AND CHEMICAL AGENTS

Most of the salmonella are killed at a temperature of 60° C. in from 15 to 20 minutes. They may persist under natural conditions for long periods of time as demonstrated in soil and water pollution studies in which typhoid bacilli have been found to survive through an entire winter in con-

taminated, frozen soil and for as long as seven days in well water. The resistance of *Salmonella* to certain dyes and chemicals is important since some of these compounds selectively inhibit the coliform organisms while allowing growth of the *Salmonella*. Brilliant green in particular inhibits coliform and dysentery bacilli, while the typhoid bacillus and other salmonella are resistant to its action. Sodium desoxycholate and selenium compounds also inhibit the growth of colon bacilli, but not the *Salmonella* under certain conditions. Sodium tetrathionate and sodium citrate favor the growth of salmonella over colon bacilli. The selective action of these compounds is used to advantage in the preparation of media for the isolation of *Salmonella* from feces.

#### ANTIGENIC STRUCTURE

The antigenic structure of the salmonella has been studied in great detail by Kauffmann (1937) and White (1926). As a result of their work the occurrence of the various known antigenic components in the cell body and flagella now serves as a basis of classification. The H, or flagellar, antigens and the O, or somatic, antigens were originally described as the heat-labile and heat-stable antigenic components, respectively. In general, the species of *Salmonella* are divided in groups on the basis of likeness with respect to O, or somatic, antigens, and the species within a group are often differentiated on the basis of differences between their H, or flagellar, antigens.

#### H ANTIGENS AND PHASE VARIATION

The H antigens are found only in the flagella. They are inactivated by temperatures over 60° C. and also by alcohol and acids. They are probably of a protein nature. For serologic testing, H antigens are best prepared by adding formalin to young, motile, broth cultures. This procedure probably fixes the flagella over the surface of



the cell in such a way that the somatic antigens are no longer exposed. As a result, agglutination is dependent on the anti-H antibodies and does not occur, or only to a slight degree, in anti-O sera. In sera containing the appropriate anti-H antibodies, H antigens characteristically flocculate in about 2 hours at 55° C. in the form of large, fluffy clumps which are easily dispersed.

A single species may contain two types of H antigens, either of which may predominate in a given instance. One of these types is referred to as the specific phase or phase-1 flagellar antigen, and the other as the group phase or phase-2 flagellar antigen. The former is shared with only a few other species or varieties of *Salmonella*. In contrast, the latter may be more widely distributed among several species. Either, phase 1 or phase 2 may contain one or more flagellar antigenic components. Any one culture may consist of organisms entirely of one phase or of organisms in both flagellar phases. Any monophasic culture usually tends to maintain this characteristic for a number of transfers but is always capable of giving rise to organisms of the other phase, especially if the culture is allowed to grow longer than 24 hours. This antigen alteration is spoken of as "phase variation." The transformation from one phase to another in a culture may be induced by growth of the culture in a serum containing antibodies against the homologous phase. Since the specific phase antigens are not entirely limited to one *Salmonella* species but may occur in several, the terms "phase 1" for the so-called specific phase and "phase 2" for the group phase have now been adopted. Phase variation can be detected only by serologic tests using sera prepared against organisms in phase 1 or phase 2.

#### O ANTIGENS

The somatic (O) antigens do not exhibit phase variation and therefore constitute a more dependable basis for classification than

the flagellar components (Kauffmann-White schema). Somatic antigens occur at the surface of the cell body (soma) in both motile and nonmotile organisms. They are resistant to prolonged heating at 100° C. and are not destroyed by alcohol or dilute acid. When mixed with sera containing appropriate anti-O agglutinins, O (somatic) antigens (prepared from nonmotile bacilli or from bacilli treated with heat or alcohol) are clumped only after long periods of incubation, e.g., 6 to 12 hours at 55° C. The bacterial aggregates so formed appear as granular masses which cannot be dispersed by shaking.

In an antiserum prepared with a motile organism as immunizing agent, the anti-H and anti-O agglutinins behave independently, and the H antibody titer is usually much higher than the O.

#### VI ANTIGENS

Recently isolated strains of typhoid bacilli often fail to agglutinate when mixed with appropriate antiserum. Felix and Pitt (1934) showed that inagglutinability is due to possession of a special somatic component called the "Vi," or virulence, antigen since cultures possessing it were more virulent for mice than ordinary O organisms. Vi antigen is thought to be present at the extreme periphery of the body of the cell and thus to prevent access of anti-O agglutinins to their homologous somatic antigens. It differs from the ordinary O antigens in being destroyed by heating for one hour at 60° C. and by dilute acids and phenol. Following repeated subculture on ordinary media, bacilli lose their Vi antigen and become agglutinable in anti-O serum. A Vi antigen apparently identical with that found in *S. typhosa* has also been recognized in other *Salmonella*, e.g., *S. hirschfeldii* and *S. ballerup*. Some other *Salmonella*, such as *S. schottmuelleri*, possess other Vi antigens specific for their type.

In 1938, Craigie and Yen discovered bac-

terio-phages active against cultures of the typhoid bacillus containing Vi antigen. These agents exhibit an unusual adaptability to the strains of bacteria on which they are propagated, resulting in the development of an extraordinary specificity for the strain of *S. typhosa*. Specific Vi phages have thus provided a valuable means of classifying typhoid bacilli, and aided etiologic studies of typhoid fever (Felix, 1947). Strain specific bacteriophages have also been adapted for use in identifying strains of *S. schottmuelleri*.

### DISSOCIATION

In addition to the flagellar phase variations, salmonella can undergo a number of alterations related to various antigenic components. Motile strains may become non-motile by losing their flagellar (H) antigens. The typical smooth to rough variation occurs frequently and results in the loss of the somatic O antigen, and the appearance at the surface of the cell of another antigen which is much less specific and causes the organism to agglutinate spontaneously in saline. The smooth to rough change may occur without loss of the Vi or flagellar antigens.

The content of any strain of *S. typhosa* in Vi antigen may vary without affecting its O or H antigens. Kauffmann (1935) has described three forms in which these organisms may be found: (1) the V form, which possesses a full quota of Vi antigen and is inagglutinable in O antiserum; (2) the VW form, in which some Vi antigen may be detected but which will agglutinate in O antiserum; and (3) the W form, which contains no Vi antigen. Since the H, O and Vi antigens vary independently, many dissociant forms can occur.

As previously mentioned, some salmonella also show a tendency to produce mucoid colonies, especially when incubated at temperatures of from 10° to 20° C.

### KAUFFMANN-WHITE CLASSIFICATION

The studies of the antigens of the salmonella by White and Kauffmann have made it possible to devise a system of classification based on antigenic patterns. The species and varieties have been arranged in groups designated A, B, C, etc., according to similarities in content of O antigens. One or more antigenic components are selected as essential for inclusion in the group. Each component of the O antigen is designated by the use of a Roman numeral. Specific sera to identify O antigens are prepared by absorption technics. Table 37 shows how the more important representative Salmo-

TABLE 37. ANTIGENIC STRUCTURE OF SOME OF THE MORE COMMON ENTERIC ORGANISMS

GROUP	TYPE	O ANTIGENS	H ANTIGENS	
			PHASE 1	PHASE 2
A	<i>S. paratyphosa</i> ...	(I), II, XII	a	
B	<i>S. schottmuelleri</i> ...	(I), IV, (V), XII	b	1, 2
	<i>S. typhimurium</i> ...	(I), IV, (V), XII	i	1, 2, 3
C <sub>1</sub>	<i>S. hirschfeldii</i> ...	VI <sub>1</sub> , VI <sub>2</sub> , VII, (Vi)	c	1, 5
	<i>S. choleraesuis</i> ...	VI <sub>1</sub> , VII	c	1, 5
	<i>S. oranienburg</i> ...	VI <sub>1</sub> , VI <sub>2</sub> , VII	m, t	
	<i>S. montevideo</i> ...	VI <sub>1</sub> , VII	g, m, s	
C <sub>2</sub>	<i>S. newport</i> .....	VI <sub>1</sub> , VIII	e, h	1, 2, 3
D	<i>S. typhosa</i> .....	IX, XII, (Vi)	d	
	<i>S. enteritidis</i> ....	(I), IX, XII	g, m	
	<i>S. gallinarum</i> ...	IX, XII		
	<i>S. pullorum</i> ....	IX, XII		
E	<i>S. anatum</i> .....	III, X, XXVI	e, h	1, 6

( ) indicate that antigen may be absent.



nella are classified according to the Kauffmann-White scheme. The members of the various groups based on the O antigen content are further differentiated into species and varieties on the basis of the components of their flagellar antigens. The flagellar antigens of phase 1 are noted by small letters. The antigens of phase 2 are designated by arabic numerals.

The Kauffmann-White system now includes over 150 types but it is questionable whether many warrant recognition as distinct species since the antigenic relationships among many are so very close. Probably many of the closely related organisms might best be considered as varieties or serologic types of a single species, as has been the practice with the serotypes of *Diplococcus pneumoniae*. In this connection, it should be pointed out that differences in the ability to ferment one or more carbohydrates may exist between strains which are apparently antigenically identical. These are designated as fermentative varieties.

The scheme of classification based on antigenic analysis is complex, but it permits the accurate etiologic diagnosis of enteric infections and the study of their epidemiology.

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

Salmonella fall into three groups with respect to their distribution and their relationship to human disease.

The first group contains those which are primarily human pathogens and includes *S. typhosa*, *S. paratyphi*, *S. schottmuelleri* and *S. hirschfeldii*. Of these *S. typhosa* is the most important. *S. schottmuelleri* is the most common in the United States (Seligmann et al., 1946), *S. paratyphi* is occasionally isolated and *S. hirschfeldii* is very rare. *S. schottmuelleri* is also found rarely in animals.

The second group is made up of organ-

isms which are primarily pathogenic for animals, including birds, but which may occasionally cause disease in man. It contains the majority of the Salmonella. The relative incidence of these species in human infections varies in different geographical areas. They are frequently named to designate the area or city where they were first isolated or to note their principal animal host. In the United States the following organisms have been most commonly isolated from patients: *S. typhimurium*, *S. choleraesuis*, *S. oranienburg*, *S. montevideo*, *S. newport*, *S. enteritidis*, *S. panama* and *S. anatum* (Bornstein, 1943). The relative incidence of infections due to any one species among the reported cases depends in large part on the number of persons involved in the outbreaks studied.

In the third group are found those which are known to be pathogenic only for animals or birds. This group has rapidly become smaller as more and more of these species have been found to cause disease in man. *S. gallinarum* is one of the most important organisms in this group.

**Salmonella typhosa** (*S. typhi*, *Eberthella typhosa*, *Bacillus typhosa*, *Bact. typhosum*, typhoid bacillus). The typhoid bacillus is found only in man and is the cause of classical typhoid fever. In animals by the oral route, it will infect only chimpanzees. By the intravenous or intraperitoneal route, *S. typhosa* in large doses will kill mice, but the size of the dose indicates that its invasive powers are not marked and that death in this host is mainly a result of the toxic action of the organisms injected. The addition of mucin to the suspension of bacteria enhances the ability of the bacillus to multiply in the mouse and makes it possible to produce death with much smaller numbers of organisms.

**Salmonella paratyphi** (*Salmonella paratyphi A*, *Bacillus paratyphosus A*, *Bacterium paratyphosum A*, paratyphoid A bacillus) is found only in man where it has been described as one cause of paratyphoid fever.

**Salmonella schottmuelleri** (*Salmonella paratyphi B*, *Bacillus paratyphosus B*, *Bacterium paratyphosum B*, paratyphoid B bacillus) is also a cause of paratyphoid fever in

man and may also produce gastro-enteritis. Although it has been isolated occasionally from animals, it does not cause disease in animal hosts under natural conditions.

*Salmonella hirschfeldii* (*Salmonella paratyphi C*) is a common cause of enteric fevers or gastro-enteritis in man in Eastern Europe and Asia but is rarely found in the United States. It is not known to be a natural pathogen of animals.

*Salmonella typhimurium* (*Salmonella aertrycke*, *Bacterium aertrycke*) is a natural pathogen of rodents causing in mice a typhoid-like disease which has a high mortality. It usually produces an acute gastro-enteritis in man.

*Salmonella choleraesuis* (*Bacterium suispestifer*), though a natural pathogen for animals, may cause an enteric fever, or gastro-enteritis in man, but usually produces localizing processes with or without a septicemia. *S. choleraesuis* also occurs in the intestine of normal hogs and occasionally in cattle and sheep. It is a prominent secondary invader in hog cholera and was once considered to be the etiologic agent until the real cause, a filterable virus, was isolated. A strain which is less virulent for hogs has been described and named *S. choleraesuis* var. *kunzendorf*. Contaminated meat may be a source of infection.

*Salmonella oranienburg* occurs naturally as a cause of epizootics in quail and chickens, and this organism is frequently isolated from dried egg products. In man, it may produce gastro-enteritis, an enteric fever or septicemia.

*Salmonella montevideo* is found in various animals and fowls (monkeys, pigs, turkeys, chickens, etc.), and also is isolated from dried egg products. In man it may cause gastro-enteritis, enteric fevers or a septicemia.

*Salmonella newport* is isolated from rats, pigs, chickens, and turkeys as well as meat and dried eggs. It causes gastro-enteritis, enteric fever or septicemia in man.

*Salmonella enteritidis* (*Bacterium enteritidis*), which resembles *S. typhimurium* in many properties, was one of the first *Salmonella* identified as the cause of disease in man by Gaertner in 1888. It includes several fermentative varieties which are sometimes given separate designations such as *S. enteritidis* var. *gärtner*, *danyz* or *essen*. Some relationship between these varieties and a specific natural host seems to exist. Strains have been isolated from horses, hogs, mice, rats, ducks and duck eggs. In man, it causes gastro-

enteritis more often than other types of clinical manifestations.

*Salmonella anatum*, isolated on several occasions from ducks, chickens, turkeys and dried eggs, has also been found in normal pigs and silver foxes. In man, it usually produces a gastro-enteritis.

*Salmonella gallinarum* is the cause of fowl typhoid and differs from other salmonella in being nonmotile. An important fermentative variant, *S. pullorum* is the cause of bacillary white diarrhea in chickens and is found in dried egg products. These organisms are commonly regarded as nonpathogenic for man, though recent evidence has suggested that *S. pullorum* may sometimes cause gastro-enteritis.

From this abridged list of representative salmonella it is apparent that most of the strains that are natural pathogens of animals can also produce disease in man. Animal products such as meat, milk or eggs are often the vehicles involved in transmission to man. These foods may come from infected animal sources or be contaminated by infected animals or man before ingestion.

## TOXINS

The endotoxins of the salmonella are toxic materials closely associated with the bacterial body which are released in solution only by autolysis and which appear identical with the cell components mentioned earlier under the name of somatic antigens. They are heat stable and neutralized only to a slight degree by immune sera. The studies of Boivin et al. (1933 a, b) and Henderson and Morgan (1938) have revealed the presence in *Salmonella* of a polysaccharide-protein-lipid complex which seems to be identical with the somatic antigen of the cell and which possesses all of the properties ascribed to the "endotoxins." Somatic antigens isolated from the various species of *Salmonella* are similar in chemical nature. The polysaccharide is responsible for the serologic type specificity of the antigen, whereas the protein seems to be identical from the antigenic point of view



in the various salmonella and shigella. The purified somatic antigen complexes elicit the production of O agglutinins, bactericidal antibodies and mouse protective antibodies when injected into rabbits or man (Morgan, 1941; Favorite and Morgan, 1942).

Somatic antigens are highly toxic. Given intravenously in man, they produce a marked febrile response. This toxic effect is not completely neutralized by specific immune serum (Morgan, 1941). Intradermal inoculation into rabbits and man produces local edema and erythema followed by necrosis. Intravenous injection into rabbits produces congestion, hemorrhagic extravasation and necrosis in various organs, particularly the liver and bone marrow. The latter shows very few polymorphonuclear leukocytes. The vascular endothelium is damaged and widespread thrombosis similar to the type characteristic of severe typhoid infections in human beings is produced (Morgan, 1943). The changes in the bone marrow and the focal necrosis in the liver are also similar to those observed in fatal human cases of typhoid fever. Following the intravenous injection in man of minute amounts of the purified somatic antigens, the individual develops a chill, fever, headache, malaise and a leukopenia with a decrease in the numbers of polymorphonuclear leukocytes. These symptoms and the leukopenia are common manifestations of typhoid fever in man.

A local intradermal injection of the somatic antigens followed after an interval by an intravenous injection results in the local hemorrhagic changes in the skin characteristic of the Shwartzman reaction.

### PATHOGENESIS

Infection with a *Salmonella* organism is almost always due to the ingestion of contaminated materials and the organisms enter the tissues through the intestine via the lymphatics. Since the number of organisms ingested apparently determines the length

of the incubation period, the latter may vary considerably in different individuals.

There are three main types of clinical manifestations of *Salmonella* infection, namely the enteric fevers, gastro-enteritis and a localizing type with foci in one or more organs accompanied by septicemia.

### TYPHOID FEVER

Among the enteric fevers the classic example is typhoid fever. The typhoid organism, unlike the other salmonella, produces only this one clinical manifestation of its invasion of the human host. The incubation period extends from seven to fourteen days. The onset is insidious, often beginning gradually with malaise, anorexia and a headache. This is usually followed by the appearance of a fever which rises in a step-like manner to an average of 104° F., with a pulse rate that tends to be slow in comparison with the height of the fever. Nosebleeds may occur at this stage of the disease. During the first week the patient usually is prostrate and may have a diarrhea, though constipation is even more common. Either condition is usually accompanied by abdominal tenderness and distension. At this time, the patient may also have a cough and bronchitis. In the first or second week, rose spots frequently appear, splenomegaly is common, and the temperature remains elevated. In the more severe cases, as time passes, the patient may become delirious and show the so-called "typhoid state." After the third week, the temperature curve shows morning remissions and returns to a normal level by a gradual lysis. A leukopenia is present in most cases characterized by a relative decrease in the number of polymorphonuclear leukocytes.

Blood cultures are often positive in the first and second weeks and less frequently during the third week; stool cultures may be positive from the beginning and often remain positive until convalescence. Organisms are often found in the urine during the

second and third week and may be excreted for a considerable period after convalescence. Cultures of bone marrow aspirations may show typhoid bacilli when blood cultures are negative. Organisms may also be found in the rose spots.

The typhoid bacillus gains access to the body through the alimentary tract where it probably multiplies during the incubation period in the lymphoid tissue of the wall of the small intestine and the regional lymph nodes. Later, organisms are often found here in the plasma cells (Adams, 1939). The intracellular position of the organism may account for the persistence of the infection in the presence of bactericidal antibodies in the blood stream and for the failure of immune serum to appreciably alter the clinical course of the disease. It is probable that the bacilli enter the blood stream from these cells at the end of the incubation period via the lymphatics and thoracic duct. The endotoxins are then released in the blood stream as the bacterial cells are lysed, producing some of the symptoms of typhoid fever. There is some evidence that the somatic antigens are present in the blood of patients during the acute phases of the disease (Dennis and Saigh, 1946).

Typhoid bacilli are found frequently in the spleen and bone marrow, and the gallbladder is invariably infected. The organisms appear to multiply in the biliary system and the majority of those which are found in the stool at certain stages of the disease, particularly in convalescence, are probably carried there with the biliary secretions. The periodicity of the discharge of the biliary contents into the intestine may account in part for the irregular results in the isolation of *S. typhosa* from the feces of any given patient over a period of time.

Relapses occur in about 10 per cent of the cases and probably represent invasion of the blood stream from the local areas of multiplication of the organisms in the lymphoid tissue, bone marrow, spleen and biliary system. The mortality rate in ty-

phoid fever is from 15 to 20 per cent. Death is due to the complications of intestinal hemorrhage or perforation in from 60 to 75 per cent of the fatal cases.

On postmortem examination, the small intestine usually shows extensive areas of ulceration, particularly in the area of Peyer's patches and hyperplasia of lymphoid tissue in the intestinal wall. Many organisms are present in the lymphoid tissue, some within plasma cells. The spleen is enlarged. The liver shows areas of focal necrosis. Organisms are almost always present in the gallbladder. Other organs may be involved such as the periosteum, bone marrow, joints and lungs. In patients with signs of meningitis, *S. typhosa* has occasionally been isolated from the spinal fluid.

#### OTHER ENTERIC FEVERS

Enteric fevers caused by salmonella other than *S. typhosa* have a shorter incubation period, 1 to 10 days, and with the exception of some of the cases due to *S. paratyphi*, *S. schottmuelleri* and *S. hirschfeldii*, are milder and less typical than typhoid fever. Fever and malaise are the dominating symptoms and last from one to three weeks. Blood cultures are often positive early in the disease, while stool cultures may be negative for one or two weeks. Rose spots are rare. The postmortem findings may or may not be similar to those of mild typhoid fever. Next to *S. typhosa*, *S. schottmuelleri* is the most common cause of enteric fever in the United States.

#### GASTRO-ENTERITIS

Following the consumption of contaminated food, gastro-enteritis caused by salmonella occurs after an incubation period of from 8 to 48 hours. This short interval suggests that large numbers of the organisms are usually ingested. The onset is nearly always sudden and may be characterized by headache and chills and often abdominal



pain. Nausea, vomiting and diarrhea follow with a rise in temperature and prostration which lasts from one to four days. The disease is more severe in infants and young children. Blood cultures are usually negative, but organisms can frequently be isolated from the feces and occasionally from the vomitus. *S. typhimurium* is the organism most commonly isolated from cases with salmonella gastro-enteritis in the United States.

### SEPTICEMIAS

In the septicemias caused by salmonella, the invasion of the blood stream is evidenced by the high remittant fever and positive blood cultures. Intestinal involvement is usually absent in adults, although in children the septicemia may occur as a complication of gastro-enteritis. Organisms may localize in any tissue of the body and may produce local abscesses in the perineal and pelvic regions, cholecystitis, pyelonephritis, endocarditis, pericarditis, meningitis, arthritis and pneumonia. *S. choleraesuis* is one of the most common organisms found in this particular type of infection. The mortality in Salmonella septicemia averages about 5 per cent but may reach 15 or 20 per cent in infections with *S. choleraesuis* (Seligmann et al., 1946).

Every *Salmonella* strain is potentially capable of producing any of these three clinical types of infection, though *S. paratyphi* and *S. schottmuelleri* tend more frequently to cause the enteric fever picture. *S. typhimurium* and *S. enteritidis* are found predominantly in acute gastro-enteritis while *S. choleraesuis* is found mostly in the septicemic type of infection. In all instances infections produced by Salmonella are more severe in infants and children than in adults. Subclinical infections in which symptoms are absent or so mild as to cause no disability may occur with Salmonella other than *S. typhosa*.

The organisms are frequently excreted in the feces during convalescence from Sal-

monella infections. When the infection is due to *S. schottmuelleri*, 20 per cent of the patients show positive fecal cultures 3 months after the disease. The proportion of patients with positive cultures begins to drop off rather rapidly after this to reach a level as low as 5 per cent at the end of the fourth month.

### CARRIERS

In some instances the salmonella may establish themselves in the tissues of the host to produce a more or less permanent carrier state after recovery from an acute infection. This is most apt to occur following typhoid fever where about 3 per cent of the cases are found to be excreting *S. typhosa* in their stools over a year after recovery from the disease. In this instance, typhoid bacilli are present in the gall-bladder or, less frequently, in the kidney tissue, where they multiply and reach the feces or urine sporadically.

The carrier state in human beings is observed less frequently with *S. paratyphi* and *S. schottmuelleri* than with *S. typhosa*, and its duration is much shorter. In a study of a large number of healthy carriers (Seligmann et al., 1946), 28 different Salmonella types were isolated with *S. schottmuelleri*, *S. typhimurium*, *S. oranienburg*, *S. montevideo*, *S. newport*, *S. panama* and *S. anatum* being the most common. Many of these carriers were contacts of cases and had exhibited no clinical symptoms of infection.

### IMMUNITY

An attack of typhoid fever usually confers immunity, though second attacks have been reported. Recovery from the disease is associated with the appearance in the blood of agglutinins and bactericidal antibodies for *S. typhosa*. These antibodies reach appreciable levels during the second and third week of the disease at a time when the typhoid bacilli are known to dis-

appear from the blood stream. Antibodies, however, may be present during the acute phase of the disease, in relapses, and at the time of progression to fatal termination (Gay, 1918). These facts strongly suggest that the presence of known antibodies is not the sole factor in recovery.

It seems probable that the circulating antibodies clear the extracellular organisms from the blood, but that the bacilli which are known to occur intracellularly in the spleen, gallbladder, bone marrow and the lymphoid tissue of the intestine are protected from their action. These bacilli multiply within the cells and release the somatic endotoxins which seem to produce tissue damage and the symptoms of general toxemia. Since the toxicity of these substances is only reduced but not completely neutralized by antibodies, they may produce their injurious effects in the presence of circulating antibody (Morgan, 1941). Humoral antibodies, therefore, appear to have a limited role in immunity to typhoid fever and the decisive factor in recovery may be the development of an increased capacity of the fixed phagocytic cells to destroy the bacteria. The persistence of resistance after antibodies have disappeared from the blood tends to support this hypothesis.

Another factor of possible importance in recovery from the disease may be the development of an increased tolerance to the toxic effects of the somatic antigens not dependent on the presence of antibodies. In man (Favorite and Morgan, 1942), increasing doses of typhoid somatic antigen may be given intravenously without corresponding increases in the severity of successive reactions. This tolerance does not seem to be correlated with antibody titer since relatively small amounts of toxin may produce toxic effects in the presence of high levels of circulating antibody. Furthermore, tolerance disappears fairly rapidly after the injections of toxin are discontinued, while the antibody levels remain elevated for much longer periods. This type of tolerance

may involve a change in the functional activity of the reticulo-endothelial system providing for a more rapid disposal of foreign material (Beeson, 1946). It is not specific for a particular somatic antigen but appears to extend to the chemically and toxicologically related but immunologically distinct endotoxins of other Gram-negative organisms (Morgan, 1947).

IMMUNIZATION

Within a few years after the isolation of *S. typhosa*, suspensions of killed bacilli were injected into human beings for the purpose of immunization. Adoption of this procedure in military personnel (Russell, 1913) was accompanied by a marked decrease in mortality due to typhoid fever (Table 38).

TABLE 38. MORTALITY FROM TYPHOID FEVER

PERIOD	UNITED STATES (Rates per 100,000)	UNITED STATES ARMY (Rates per 100,000)
1906-1910	25.6	26.37
1911-1915	16.6	3.24 *
1916-1920	11.1	5.08 †
1921-1925	7.6	0.4
1926-1930	5.1	1.19
1931-1935	3.5	0.59
	93 CITIES ‡ IN THE UNITED STATES	UNITED STATES ARMY IN EUROPEAN THEATER §
1942-1945	0.25	0.06

\* 1911—Compulsory immunization introduced into U. S. Army.  
† 1916—Mexican Border Service; 1917-1919 World War I.  
‡ Total population of 38,060,662.  
§ Army had maximum strength of 3,064,562 in this theater.

Although available data tend to support the view that vaccination significantly reduces the incidence of this infection, it is often



difficult to assess the results since general conditions of sanitation began to improve both in military and civil practice at about the same time that vaccination was introduced. In consequence, the use of the comparative incidence of typhoid fever, during consecutive periods of years as an indication of the efficacy of vaccination, is invalid unless the sanitary conditions are known to be identical in these successive periods. In a study by Callender and Luipold (1943) in which a standard vaccine had been used for the immunization of a group of men who were subsequently exposed to a common contaminated water supply, the incidence of typhoid fever among the nonimmunized group was 7 per cent as compared with 1.1 per cent in the vaccinated group. These results suggest that the protection conferred by vaccination is only relative. Since the infecting doses may be very large, in some instances, the immune mechanism may be overwhelmed thus explaining the failure of the vaccine to give complete protection under all conditions.

The vaccine commonly used consists of a saline suspension of typhoid organisms in the smooth phase killed by heating for 1 hour at 56° C. The suspension is standardized to contain one billion organisms per ml. Tricresol or an organic mercurial is added as a preservative.

Grinnell (1932) studied the immunizing potency of the various dissociants of the typhoid bacillus and found that vaccines prepared from rough strains had little value. This is confirmed by the findings (Felton and Wakeman, 1937) that the O somatic antigens which are lost in the S→R dissociation are the essential immunizing constituents of salmonella. Still more recently recognition that the Vi antigen is destroyed by heating has led to the use of an alcohol inactivated vaccine, in which the Vi antigen is preserved. However, it has not yet been demonstrated that the Vi antigen plays a role in protection.

Since the O antigens can now be prepared

in relatively pure form, they have been used in experimental immunization in man. In very small amounts, they produce somewhat higher circulating antibody titers than the whole bacterial vaccines with less unpleasant toxic reactions (Morgan, 1945). Another type of antigenic preparation, called endotoxoid, is prepared by causing dissolution of the bacterial cells by repeated freezing and thawing, and detoxification with formalin. It has been described by Grasset (1936) as producing an adequate immune response.

During periods when sanitation breaks down, a wider range of protection may be desired, and typhoid vaccine is often supplemented by the addition of suspensions of heat-killed *S. paratyphi* and *S. schottmuel-leri*. This preparation is designated T.A.B. vaccine. In certain geographic areas it is modified to include *S. hirschfeldii* (T.A.B.C. vaccine). These vaccines are given in the form of 3 injections of 0.5 to 1.0 ml. subcutaneously and reinoculation with 0.5 ml. every three years. The administration of 0.1 ml. intracutaneously as a booster dose produces fewer undesirable reactions; yet it appears to give adequate antibody titers. It has been suggested that the intracutaneous route might be used also for primary immunization. Administration of vaccines by the oral route has not been shown to elicit an adequate antibody response.

The value of anti-typhoid immune serum used prophylactically in individuals known to have been exposed to typhoid fever has not yet been established.

## DIAGNOSIS

The diagnosis of infection with a *Salmonella* organism depends upon isolation and identification of the causative agent (e.g., from blood, feces or urine) or upon the demonstration of a rise in titer of specific circulating antibodies for a given organism.

Isolation of the organism from the circulating blood is the most conclusive method

of bacteriologic diagnosis. It is most likely to be successful in the earliest phases of the disease and in the enteric fever or septicemic types of clinical disease. In typhoid fever, positive blood cultures are obtained in 80 to 90 per cent of cases during the first week. Blood cultures are usually negative in cases of salmonella gastro-enteritis.

Organisms may be isolated from the feces in typhoid fever or in salmonella gastro-enteritis during the acute stage of the disease and for a variable period thereafter. In typhoid fever the number of positive fecal cultures may increase during the second week. Organisms may also be isolated from the vomitus or from the contaminated food ingested in the cases of gastro-enteritis. In *S. typhosa* infection as many as 25 per cent of the cases have positive urine cultures more commonly in the later stages of the disease. Salmonella may be found in the urine of patients with the septicemic type of infection when the organisms have localized in the kidney.

Isolation of salmonella from the feces presents a special problem because of the huge number of normal fecal organisms. [It is important to culture the fecal specimen as soon as possible to prevent overgrowth by the normal fecal flora and a direct rectal swab may be a useful adjunct in obtaining a fresh specimen (Hynes, 1942).] A variety of materials have been added to special media to aid in the isolation of intestinal pathogens. They fall into several categories: (1) agents which inhibit the growth of normal intestinal bacteria, e.g., dyes like brilliant green, selenium salts and bile salts; (2) agents which favor the growth of *Salmonella* over the coliform organisms, e.g., sodium tetrathionate and sodium citrate; (3) substances which give to *Salmonella* colonies distinguishing characteristics, e.g., lactose with necessary dye indicators to color the lactose-fermenting colonies of the coliform group, sulfite which is reduced to sulfide by many *Salmonella* and gives a

black color to the colony in the presence of iron salts.

The fresh stool specimen should be streaked immediately on a selective medium, e.g., SS agar (Mollov et al., 1943), or sodium desoxycholate citrate agar (Leifson, 1935) and on a nonselective medium such as EMB or Endo. The nonselective medium is included to pick up the rare species of *Salmonella* that do not grow well on the selective media. For use in the isolation of *S. typhosa*, the bismuth sulfite selective medium is of particular value (Wilson and Blair, 1931). At the same time, the specimen should be inoculated into an enrichment medium which allows the pathogens to multiply and which inhibits *E. coli*, e.g. brilliant green or tetrathionate broth (Hynes, 1942). After incubation for from 12 to 24 hours, this enrichment culture is streaked on selective and nonselective agar plate media. Suspected colonies are subcultured and identified by biochemical reactions and by agglutination tests with specific absorbed sera. Further species identification of *Salmonella* can be obtained by sending the culture to one of the *Salmonella* Centers.

#### SEROLOGIC DIAGNOSIS

Agglutinins for the causative organisms can usually be demonstrated in the circulating blood of patients from one to two weeks after the onset of typhoid and enteric fever, septicemia, or gastro-enteritis of the more severe types. In mild diarrheas, the agglutination reaction may not become positive until after the symptoms have subsided, or, in some instances, it may remain negative. Tests should be carried out for both O and H agglutinins, but O agglutinins appear to be of more significance since some cases fail to develop H agglutinins. Moreover, O agglutinins are of more diagnostic value because they disappear more rapidly than H agglutinins following vaccination. Finally, O antigen relationships among the



various *Salmonella* broaden the range of the test for the initial detection of infection.

The antigens used in the agglutination test should be so composed as to give an adequate coverage for all of the principal *Salmonella* species occurring in the particular geographic area. After this preliminary screening, further testing can be carried out to give definite diagnosis of the species involved. In the United States *S. typhosa*, *S. schottmuelleri* and *S. choleraesuis* are commonly used for screening agglutination tests. It is not necessary to include a Vi antigen for the diagnosis of typhoid fever since Vi agglutinins rarely appear in the blood of patients in the absence of H or O agglutinins. Also, Vi agglutinins may not be detectable in the serum of patients known to have typhoid fever. Typhoid somatic antigen has been demonstrated in the serum of patients during the disease (Dennis and Saigh, 1946) by the use of specific hyperimmune serum. This technic and the detection of somatic antigen in the urine of patients have been suggested for use in the early diagnosis of the disease. The detection of agglutinins in the feces of patients (Harrison and Banvard, 1947) has recently been suggested as a diagnostic method.

In the Widal or agglutination test, the demonstration of a rising titer of specific agglutinins is accepted as definite evidence of infection with the particular *Salmonella* strain. However, if only a single specimen is available, an O agglutinin titer of more than 1:50 during the first 10 days of illness is considered strong presumptive evidence if the patient has not been vaccinated within one or two years. In a patient with a history of previous inoculation with typhoid vaccine, the fact that H agglutinins tend to persist for a number of years following immunization while O agglutinins fall to low levels in six months and usually disappear in a year makes the O agglutinin titer more valuable in diagnosis. However, several other factors should also be considered in

the interpretation of a single agglutination titer. Thus, the level of agglutinins in the serum of normal persons in the particular geographic area is important and in all instances the stage of the disease at which the serum specimen was taken should be

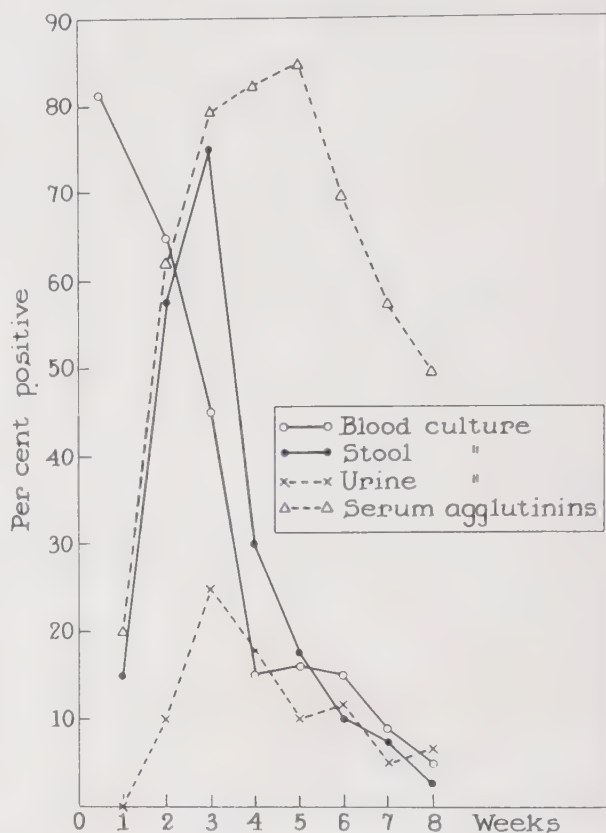


CHART 11. Results of serum agglutination tests and of blood, stool and urine cultures on patients during the course of typhoid fever.

noted. Since antigen preparations differ in their sensitivity to the agglutinating action of sera, the activity of each new antigen should be evaluated with a standard serum.

## TREATMENT

The treatment formerly used in typhoid fever or other salmonella infections was mainly supportive and aimed at maintaining the fluid balance and nutritional state of the patient. More recently several specific therapeutic agents have been employed in-

cluding immune sera, bacteriophage and antibacterial drugs.

Felix has prepared a concentrated anti-typhoid immune serum containing Vi and O antibodies which has been reported to decrease the severity of toxic symptoms and to shorten the duration of the disease (Landor, 1941), but adequate series of treated and control cases are not yet available. While the earlier experiences with bacteriophage therapy in typhoid fever were disappointing, favorable results have been recently reported with strain specific Vi bacteriophages. The sulfonamide drugs have apparently had some beneficial effect on certain salmonella infections, but their use in typhoid fever has proved disappointing. Combined therapy using sulfonamides with larger than ordinary doses of penicillin seems to have given some evidence of beneficial effects. Streptomycin has had a limited trial in typhoid fever and in other *Salmonella* infections without any clearly beneficial results in spite of the fact that the organisms are sensitive to the action of the drug in vitro (Keefer, 1946). Streptomycin given orally markedly reduces the number of typhoid bacilli as well as of coliform organisms in the stools. The bacteria reappear, however, when the therapy is discontinued.

Some studies with the antibiotic, chloromycetin, have indicated that it may be a valuable therapeutic agent in the treatment of salmonella infections.

## EPIDEMIOLOGY

The source of all salmonella infection is the reservoir of organisms living in the tissues of human beings or animals. Infection occurs through food, milk or water contaminated with infected feces or urine or by the actual ingestion of the infected animal tissues. The hosts which harbor the organisms may be clinically recognized cases or sick animals, subclinical cases, or carriers. The

two latter groups are the most important since they are usually unrecognized. Infection with most salmonella apparently requires the ingestion of large numbers of organisms, but in the case of *S. typhosa* relatively few bacilli are sufficient to cause typhoid fever. This difference in infectivity probably accounts for the fact that lightly contaminated materials like water or shellfish can be the source of typhoid infection while most other salmonella infections are caused by heavily contaminated food in which the organisms often multiply before ingestion.

Water may be contaminated with infected feces by cross connections between water and sewage pipes, seepage of surface water into wells or surface contamination of shallow wells. Epidemic outbreaks of typhoid fever involving entire communities may follow a breakdown of the water purification system during a flood, for example. Outbreaks involving fewer individuals may be caused by contaminated milk not pasteurized or, more rarely, contaminated after pasteurization. In these instances, the distribution of cases parallels that of the delivery of the milk. Oysters and other shellfish may also cause smaller outbreaks when taken from water contaminated with sewage.

Endemic typhoid fever and infections with other salmonella are now much more common than epidemic typhoid fever in parts of the world where sanitary measures are applied to protect water and food supplies. In these instances, infection is usually due to contamination of food by a human being or animal excreting the organisms; or to the ingestion of the improperly cooked meat of an infected animal; or of foods containing contaminated egg products. In some instances flies carry infected excreta to the food or even become infected themselves with the bacilli and deposit them on the food. Milk and dairy products such as cream, custard, ice cream and cheese have



been involved in the spread of typhoid fever and other *Salmonella* infections. The bacilli can grow in foodstuffs at warm temperatures without producing any detectable changes in appearance or taste as evidence of their presence. Among foods that may be prepared from the tissues of animals infected with *Salmonella* bacilli are meat products like sausage and luncheon meats, which are often insufficiently cooked. Eggs are often implicated, and positive *Salmonella* cultures have been obtained in as many as 30 per cent of a series of preparations of powdered eggs, most of which contained *S. pullorum*, although *S. oranienburg*, *S. typhimurium* and *S. montevideo* were also found. Ducks are known to harbor *S. typhimurium* and *S. enteritidis*, which have been found in their eggs.

From 2 to 5 per cent of recognized clinical cases of typhoid fever become chronic carriers and continue to excrete *S. typhosa* in their stools and, in some instances, in their urine, for years. They constitute the main source of infection in this disease, although ambulant cases also spread the organisms. Since over 90 per cent of typhoid carriers show Vi agglutinins in their serum, this provides a valuable screening test for their detection. Once proven to shed bacilli from their urine or feces, these persons must be isolated until rid of the carrier state by procedures such as cholecystectomy or drug therapy. Disappearance of Vi agglutinins suggests that the carrier state has been eradicated but this is proven only by repeated negative fecal and urine cultures.

Chronic carriers who excrete organisms for over a year are found much less often following infections with *S. paratyphi* and *S. schottmuelleri* and even more rarely with other organisms such as *S. typhimurium*. With other salmonella, the most important sources of infection are the temporary convalescent carriers, who may continue to excrete the bacilli for from 4 to 16 weeks after their illness, and the ambulant, subclinical cases who excrete the organisms in their

stools (Seligmann et al., 1946). In any outbreak, there may be a large number of these apparently normal contacts who are excreting the bacilli and therefore serve to spread the infection.

## CONTROL MEASURES

The control of *Salmonella* infections is directed at: (1) the elimination of the sources of infection, (2) the detection and elimination of the modes and vehicles of transmission, and (3) measures to increase the resistance of the susceptible host. The first two aspects of control are the most important and are usually relied on entirely except under circumstances in which control of the environmental factors becomes more difficult, as in time of war when the third aspect may assume a more critical role.

In the case of typhoid fever elimination of sources of infection is largely a question of detection and isolation of the chronic human carrier. This measure is also valuable in the prevention or spread of other salmonella infections. In all instances, the infected patient must be recognized, isolated and his excreta carefully disposed of. Bacteriologic examinations should be made of the stools in all cases of diarrhea and all salmonella infections should be reported. The detection and isolation of the ambulant cases in an outbreak is a valuable adjunct in control when this procedure is feasible. Ideally, any individual from whom positive cultures of feces or urine have been obtained should be isolated until several successive negative cultures have been obtained.

Adequate inspection of animals slaughtered for meat is of value in limiting the consumption of meat from sick animals. However, if the disease is not active, the detection of infected animals may be very difficult. Inspection and supervision of other animal food products also serves to reduce contamination.

Spread of the infection can be halted by methods directed at the vehicles and modes of transmission. Modern sanitation methods such as proper sewage disposal, selection of unpolluted sources of water supply with its subsequent filtration and chlorination, and the pasteurization of milk have been largely responsible for the dramatic decrease in the incidence of typhoid fever.

The contamination of food may be prevented by the exclusion as food handlers of persons with diarrheas and by the elimination of rodents and flies from premises where food is prepared. Careful handling of food after preparation, adequate refrigeration of uncooked foods, adequate cooking of meats, and of eggs and egg products all serve to reduce *Salmonella* infection.

Active immunization with typhoid and *Salmonella* vaccines is best regarded as an emergency measure to be used under conditions of great exposure: for example, among the hospital staffs of contagious disease hospitals, among soldiers or travelers in countries where the diseases are still endemic due to lack of sanitary developments; or when sanitation breaks down as

in catastrophes or periods of war. Vaccination is only a poor substitute for sanitary control since there is no evidence that it alone can stamp out intestinal diseases; however, it seems to reduce the incidence and fatality of *Salmonella* infections. Cases do occur among vaccinated individuals (Syverton et al., 1946), especially when the infective dose is large. This was demonstrated in the prisoner of war camps in World War II where vaccination apparently was effective in aiding in the control of disease until the camps became severely overcrowded. Prophylactic vaccination during an epidemic is of value mainly in preventing secondary cases among contacts of infected individuals. If the outbreak is explosive, as in the case of a water-borne epidemic of typhoid fever where the source can be discovered and controlled early, immunization of the entire community is not necessary since it can have no effect on individuals in the incubation period of the disease and is unnecessary for those who have escaped infection, with the exception of those in direct contact with the patients (Topley, 1938).

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## 18

# Bacillary Dysentery and the Shigella

### INTRODUCTION

Organisms of the genus *Shigella* are nonencapsulated, nonmotile Gram-negative rods, approximately twice as long as broad. They grow best under strictly aerobic conditions, although some growth occurs anaerobically. They ferment various carbohydrates with production of acid, but no gas; they fail to liquefy gelatin, to produce hydrogen sulfide or acetyl methyl carbinol, and to utilize citrate. Many species are related antigenically, and there is some sharing of common antigens with members of other genera. Their natural habitat is restricted to the gastro-intestinal tract of warm-blooded mammals, in contrast to the more ubiquitous distribution of members of the genera *Salmonella* and *Escherichia*. Some species of *Shigella* are pathogenic for man and represent the etiologic agents of bacillary dysentery. Other species occupy a doubtful position, while a third group is nonpathogenic in the light of our present knowledge.

### HISTORY

Brief historic accounts are given by Rogers (1913), Felsen (1945), and by Topley and Wilson (1946).

The clinical entity of bloody or mucous diarrhea accompanied by straining and tenesmus was recognized by ancient authors as long ago as the fourth century, B.C. Herodotus ascribes the defeat of the Persian Army in 380 B.C. in part to dysentery. The clinical and epidemiologic descriptions furnished by Hippocrates in the same century suggest that the disease was

well known in Greece and that the bulk of it was probably of bacillary rather than amebic origin. Until the recent advent of effective field sanitation the conditions associated with military operations have been particularly conducive to the spread of enteric disease, and it is not surprising that outbreaks of dysentery have been a frequent accompaniment of military campaigns. In recent times the Crimean War, the American Civil War, the South African War, and the Gallipoli campaign of World War I have furnished invaluable clinical and epidemiologic data to students of the disease. In civil life jails and asylums have long been notorious for outbreaks of dysentery; overcrowding and poor sanitary facilities appear to have been the common factors.

In spite of the many excellent clinical accounts of the disease which have been published since the time of Herodotus, it was not until the closing years of the nineteenth century that bacillary and amebic dysentery were separated on clinical epidemiologic and etiologic grounds. The latter disease occurs sporadically rather than in epidemics, pursues a chronic rather than an acute course, is frequently complicated by hepatic abscesses if specific therapy is not promptly instituted, and presents the typical pathologic findings of shallow undermining ulcers in the large bowel, and an



exudate which is mononuclear in character. Although the causative organism, *Entamoeba histolytica*, was probably discovered as early as 1859 it was not until the turn of the century that its etiologic relation to the clinical disease was firmly established.

Attempts to incriminate a specific bacterial agent as a cause of dysentery met with failure until the work of Shiga in 1898. He was able to isolate the organism now known as *Sh. dysenteriae* from the feces and intestinal walls of patients suffering from clinical dysentery. He recovered the organism during the acute phase of disease with considerable frequency but only rarely during convalescence, and never from healthy individuals. Further proof was furnished by the finding of specific agglutinins against the organism in the blood of patients suffering, convalescing or recently recovered from the disease, but not in the blood of healthy individuals (Shiga, 1898). Two years later the first type of *Sh. paradysenteriae* was isolated by Flexner in the Philippines in similar fashion from the feces of patients suffering from dysentery. The first adequate description of *Sh. sonnei* was given by Sonne of Denmark in 1915. *Sh. ambigua* was isolated by Schmitz in a Roumanian prisoner of war camp during an outbreak of dysentery in 1917. Much of our information concerning the para-Shiga group of dysentery bacilli comes from the recent work of Sachs in India. These organisms, which resemble either *Sh. dysenteriae* or *Sh. ambigua* in their biochemical reactions although they are antigenically unrelated, have been reported from North Africa and the United States as well as from India. They have received no official species designation as yet. Of the nonpathogens, *Sh. alkalescens* was first identified, in 1918, by Andrewes, who at the same time applied the name *Sh. dispar* to another nonpathogenic species which includes the types *Sh. ceylonensis* and *Sh. madampensis*, described by Castellani several years earlier.

## MORPHOLOGIC AND BIOCHEMICAL CHARACTERISTICS

The review of Neter (1942) may be consulted for further details on this matter. Members of the *Shigella* group are slender, nonsporing Gram-negative rods approximately  $1\ \mu$  by  $0.5\ \mu$ , and as such bear a close morphologic resemblance to other enteric and related bacteria. On primary isolation they may be so short as to appear coccobacillary in form. They are nonmotile and unencapsulated.

They are facultative anaerobes, growing best at a temperature of approximately  $37^{\circ}\text{C}$ . Their metabolic requirements are relatively simple, and the ordinary laboratory media will support their growth. Their ability to grow in the presence of various bile salts in contrast to some strains of coliform bacilli is of practical importance in cultural procedures for their isolation from the gastro-intestinal tract. They do not liquefy gelatin nor do they produce hydrogen sulfide; they are unable to utilize citrate. All members of this group fail to produce acetylmethyl carbinol but are able to reduce nitrates to nitrites. Some species produce indole.

Like other nonsporulating organisms, their resistance to physical and chemical agents is not remarkable. A temperature of  $55^{\circ}\text{C}$ . sustained for one hour will kill them, as will exposure to 1 per cent phenol for 30 minutes. They may, however, survive in sea water for at least 3 days, and for considerably longer in the dried state, particularly if they are kept in the dark. Under natural conditions in the stool their survival period appears to be short, presumably because of their sensitivity to the acidity produced by the growth of organisms. Thus, it is important to culture stool samples as promptly as possible.

Individual species of *Shigella* ferment a variable number of carbohydrates with the production of acid but, with two excep-

tions, no gas. Their fermentation reactions are sufficiently constant and characteristic to be of use in their identification and classification. All ferment glucose, and none salicin. On the basis of their ability to ferment mannitol they may be divided into two primary groups: (1) *Sh. sonnei*, *Sh. dispar*, *Sh. alkalescens* and *Sh. paradysenteriae* (with the exception of a few strains of the Newcastle type) are mannitol fermenters. (2) In the nonmannitol fermenting group fall *Sh. dysenteriae*, *Sh. ambigua* and the members of the para-Shiga group. *Sh. sonnei* and *Sh. dispar* ferment lactose; the fermentation is characteristically slow, however, and is not apparent for several days. In the case of *Sh. sonnei* at least this property is due to the development of lactose fermenting papillae on the original lactose negative colonies grown out on primary isolation (Glynn and Starkey, 1939). With the exception of these species, the members of the genus *Shigella* resemble the other enteric pathogens in their inability to ferment lactose. *Sh. alkalescens* usually ferments dulcitol while most types of *Sh. paradysenteriae* do not—a useful differential point. Two types of *Sh. paradysenteriae* form exceptions to the rule that dysentery bacilli are anaerogenic: the so-called Newcastle bacillus may produce a small quantity of gas in glucose and dulcitol while the closely related Manchester bacillus may produce gas in mannitol as well. The ability of *Sh. ambigua* to produce indole as compared to the failure of *Sh. dysenteriae* to do so helps to differentiate these organisms. The para-Shiga bacilli may resemble either *Sh. dysenteriae* or *Sh. ambigua* in their fermentation and other biochemical reactions. While these various biochemical reactions are of considerable usefulness in the tentative differentiation and identification of species and types, the final identification of the various shigellae depends upon the study of their antigenic structure.

## ANTIGENIC STRUCTURE

The antigenic pattern of the *Shigella* is complex. All species tend to be antigenically heterogeneous, some much more so than others. Serologic overlapping between different species occurs; furthermore, antigens found in certain species have been detected in other enteric bacilli. A further source of trouble is the occasional instance in which there is a lack of correlation between serologic and biochemical characteristics. In such a case, antigenic structure is usually accepted as the final criterion for purposes of classification.

*Sh. dysenteriae* is, relatively speaking, antigenically homogeneous—that is, an antiserum prepared against one strain will agglutinate all other strains to approximately the same titer but will not agglutinate other species. Freshly isolated *Sh. ambigua* strains are antigenically homogeneous also, but instances have been reported where antigenic variants have appeared after repeated subculture in the laboratory. Although there may be some cross reactions between *Sh. dysenteriae* and *Sh. ambigua* they are relatively unimportant. The para-Shiga group is antigenically heterogeneous since at least 5 types have been recognized (Wheeler and Stuart, 1946); significant cross reactions with members of other species have not been reported. *Sh. sonnei* is heterogeneous as two antigenic types have been recognized (Wheeler and Mickle, 1945); both types are essentially smooth in character, although they may be differentiated by their colonial morphology. Cross reactions with certain types of *Sh. paradysenteriae* have been reported. Comparatively little is known of the antigenic structure of *Sh. dispar*; however, four types have been distinguished (Carpenter, 1943), and the species is presumably heterogeneous. The antigenic structure of *Sh. alkalescens* is complex, and has long been a matter of controversy. Although the individual strains of this species appear to



be serologically identical the careful studies of Stuart et al. (1943) have shown that these organisms contain at least five antigenic components: "A" is species specific, "B," "D" and "E" are found in coliform and paracoliform bacilli, while "C" is found in *Sh. paradysenteriae* as well.

*Sh. paradysenteriae* presents the most complicated picture, and, in spite of the efforts of many investigators, the picture is still not clear. The early work of Andrewes and Inman (1919), led to the concept of four antigens (v, w, y and z) being present in any given strain in varying proportions; strains were identified and labelled according to the predominant antigen. The investigations of Boyd (1938, 1940) have altered this concept, however; he identified 6 distinct types in the Flexner sub-group, each characterized by the possession of a common group antigen and of a type-specific antigen. The so-called "Boyd" sub-group contained types characterized by the presence of specific antigens limited to each type, and the absence of any group antigen common to all members. This concept has been confirmed and extended by Wheeler (1944), who has identified at least 6 types within the Boyd subgroup. Although other possibilities have been suggested by the work of Weil and his collaborators and of Goebel, Perlman and Binkley (who have approached the problem from the chemical angle), it offers the most reasonable serologic classification of the group at this time. For references Weil's recent review (1947) may be consulted. "New" members of this group have been reported recently. At present the separation of the various members of *Sh. paradysenteriae* into serologic types has its chief practical value in epidemiologic studies.

#### SOMATIC ANTIGENS AND TOXINS

The chemical structure of the somatic antigens of members of the *Shigella* group has been studied most fully in *Sh. dysen-*

*teriae* (Boivin, 1940; and Morgan and Partridge, 1941), and in *Sh. paradysenteriae* (Binkley, Goebel, and Perlman, 1945). These antigens appear to be complex molecules containing lipid, carbohydrate and protein components. In the case of *Sh. dysenteriae* the diethylene glycol extraction of acetone-treated bacterial cells yields a nontoxic polysaccharide haptene which confers serologic specificity upon the smooth organism and which is lacking in the rough variant, a nontoxic and nonantigenic lipid fraction, and a conjugated protein, which is toxic, weakly antigenic, and apparently identical with the analogous fraction of *S. typhi*. In the case of *Sh. paradysenteriae* extraction with aqueous pyridine has given good yields of antigen of the same general chemical nature. The toxic properties of the various components vary with the methods of extraction. Weak-acid hydrolysis yields a nontoxic carbohydrate fraction which acts as a haptene rather than an antigen, a weakly antigenic but fully toxic protein component and a nontoxic, nonantigenic phospholipid. If alkaline degradation is employed, however, the toxic factor is associated with the carbohydrate rather than the protein fraction. As compared to the true exotoxins, the relative toxicity of even the more purified preparations of the somatic antigen is not great.

The complete somatic antigens of the *Shigella*, separated by chemical methods, are highly antigenic, relatively heat stable, and show pharmacologic and physiologic properties resembling those of the analogous antigens of other Gram-negative bacilli. Injected parenterally into rabbits, mice or guinea pigs they cause diarrhea, weight loss, inflammation and even hemorrhage and necrosis of the gastro-intestinal tract. These pathologic lesions closely resemble those found in cases of clinical bacillary dysentery and it would seem reasonable to suppose that the characteristic signs and symptoms of the clinical disease in man were due in part at least to the release of "endo-

toxin" (i.e., the toxic component of the somatic antigen) in the gut. This theory, however, fails to explain the inability of coliform bacilli, which possess a closely related toxic component, to cause acute gastro-enteritis.

In common with other members of the genus *Shigella*, the smooth forms of *Sh. dysenteriae* contain a heat stable endotoxin which appears identical with the somatic antigen. In addition, both smooth and rough forms of this organism produce a specific thermolabile substance which is fully antigenic and highly toxic for mice, rabbits and guinea pigs. It is usually referred to as "Shiga exotoxin" because it may easily be separated from the cell bodies. There is, however, no evidence that it is excreted by living cells, and methods which favor the autolysis of cells give the highest yields. Several methods for the rapid production of this toxin have been described (Dubos and Geiger, 1946). It is protein in nature and may be purified and concentrated by selective precipitation (Anderson et al., 1945). Injected into rabbits, mice, or guinea pigs it causes paralysis of the limbs, diarrhea and death. It stimulates the production of a specific antitoxin which neutralizes the lethal effect of the toxin, combining with it according to the law of multiple proportions. It may be detoxified by formalin at an alkaline pH (Farrell and Ferguson, 1943) or by ultraviolet radiation (Branham and Habel, 1946), and the resulting toxoid is still antigenic. The injection of this material into human volunteers has produced local and systemic reactions comparable to those following vaccination against typhoid fever (Farrell et al., 1944). Since there is no evidence that it is excreted by living cells, but rather that its production follows upon the death and autolysis of the bacteria, it is probably not a true exotoxin. Its relation to the pathogenesis of clinical dysentery in man associated with this specific organism is uncertain. With the possible exception of *Sh. ambigua*, there is

no evidence that other species of *Shigella* produce similar toxins.

## VARIATION

In 1921 Arkwright described smooth and rough forms of *Sh. dysenteriae*. These observations have been confirmed and extended to other members of the genus (Waler, 1935). The S to R variation is associated with the loss of the so-called endotoxin, the toxic factor associated with the somatic antigen. Strains freshly isolated from human cases of clinical dysentery are usually in the smooth phase. The ability of rough forms to cause clinical disease remains undetermined.

Phase variation has been described in the case of *Sh. sonnei* (Wheeler and Mickle, 1945). Phase 1 and phase 2 occur in stool cultures from *Sh. sonnei* infections. Although both are "smooth" in character they may be distinguished by their characteristic colonial morphology and by antigenic analysis. On subculture phase 1 tends to become the antigenically broader phase 2; old laboratory strains frequently show a third colonial variant with the characteristics of a true R form.

Boyd (1938) has described a somewhat similar type-group variation among certain strains of *Sh. paradysenteriae*. Two types of colonies were noted upon subculture of a given strain; the first contained both the type-specific and the group-specific antigens, the second only the latter, which, however, retained the characteristics associated with smooth strains. This type-group variation appeared to be irreversible inasmuch as there was no tendency to regain the type-specific antigen once it was lost. Freshly isolated strains usually contained both the type-specific and the group-specific antigens.

## BACTERIOPHAGE

The Gram-negative bacteria inhabiting the gastro-intestinal tract of man are usu-



ally susceptible to bacteriophage action, and specific phages effective against most members of the genus *Shigella* have been isolated. Burnet and McKie (1930), working with various types of *Sh. paradysenteriae*, have shown that these strains show characteristic differences in their phage sensitivity. Antigenically similar strains showed practically identical reactions toward the series of phages tested. Furthermore, certain phages lysed only the smooth organisms, while others were effective against the corresponding rough forms. Bacteriophage typing may prove to be a useful tool in the identification of various species and strains of dysentery bacilli.

#### NATURAL HABITAT AND RANGE OF PATHOGENICITY

The natural habitat of the dysentery bacilli is the gut of mammals. Man is the species chiefly affected, although monkeys raised in captivity may excrete organisms in their stools; dogs have rarely been found to be infected. The naturally occurring disease, however, is limited to man and perhaps monkeys, and these are the only species that develop clinical manifestations following the oral ingestion of the specific organisms. As yet no significant animal reservoir has been discovered. Contamination of food and water by human fecal discharges may, of course, lead to the isolation of dysentery bacilli from these sources.

#### SUGGESTED CLASSIFICATION

The classification of the *Shigella* is neither easy nor entirely satisfactory. A combination of biochemical and antigenic characteristics offers the most practical criteria for classification at present. New strains are reported from time to time, and careful study is required in order to determine whether or not they deserve the rank of species. Assuming the relative permanence of serologic and biochemical characteristics

of a given organism, a useful provisional classification is as follows (modified from Topley and Wilson, 1946):

1. Nonmannitol and nonlactose fermenting organisms.
  - a. *Sh. dysenteriae* (antigenically distinct and homogeneous).
  - b. *Sh. ambigua* (antigenically distinct and homogeneous).
  - c. The para-Shiga group (probably contains at least 5 antigenic types).
2. Mannitol fermenting and nonlactose fermenting organisms.
  - a. *Sh. paradysenteriae*.
    - (1) The Flexner group. Six types sharing a common group antigen but characterized by the possession of additional type specific antigens.
    - (2) The Boyd group. Six types containing type specific antigens only.
  - b. *Sh. alkalescens*. One antigenic type, containing a major species-specific antigen.
3. Mannitol and lactose fermenting organisms.
  - a. *Sh. sonnei*. Two antigenic types recognized.
  - b. *Sh. dispar*. Four antigenic types recognized.

This classification is both provisional and arbitrary. As our knowledge of the dysentery bacilli increases, it should be possible to develop a more logical one.

#### PATHOGENESIS

Dysentery bacilli usually reach the gastrointestinal tract through the medium of infected food or water. In direct contrast to typhoid fever no septicemic phase occurs, and the organisms remain limited to the gut wall. The essential pathologic process is an inflammatory one which always involves the large bowel and occasionally the terminal ileum as well. Inflammation of the mucous membrane of the bowel wall is followed by necrosis, which in severer cases goes on to actual ulceration which may reach the muscularis mucosa. In contrast to amebic dys-

entery, the edges of the ulcers remain sharp, and undermining does not occur. In all but mild cases some degree of hemorrhage takes place. The intervening mucosa is inflamed and edematous, and microscopic examination of the bowel wall shows it to be infiltrated with polymorphonuclear cells. As the process subsides, granulation tissue replaces the ulcerative lesions; in severe cases scar tissue develops. It is believed that the pathologic changes follow upon the local irritative action of the heat-stable endotoxin released by the autolysis of the bacterial cells which are found in large numbers on the floor of the ulcers, and frequently in the inflamed mucosa as well. Direct experimental proof of this is lacking, although the parenteral injection of large amounts of dysentery bacilli autolysates into laboratory animals gives rise to somewhat similar lesions. Coliform bacilli fail to cause gastrointestinal disease, although they possess an analogous endotoxin.

The classic clinical picture of bacillary dysentery is dominated by diarrhea, abdominal pain and fever. The incubation period is variable but may be as short as 24 hours. Abdominal discomfort and cramps (often described as "griping") are the first symptoms, and usually come on suddenly. They are followed shortly by diarrhea, which, in all but the milder cases, is accompanied by straining and tenesmus. The stools are liquid almost from the start; large amounts of mucous are passed, and blood in severer cases. The diarrhea and the abdominal cramps reflect the acute inflammation of the large bowel; straining and tenesmus furnish evidence that the process involves the rectum as well. The fever which accompanies the typical case is presumably due to the absorption of toxic products from the gut. The disease tends to be self-limited, and uncomplicated recovery is the general rule. Cases of chronic relapsing dysentery are usually of amebic origin. Complications are rare, and, although some authorities feel that most cases of "idiopathic ulcerative colitis"

are sequelae of chronic bacillary dysentery, the relationship between the two conditions remains to be determined. A small proportion of recovered patients become chronic carriers of dysentery bacilli. These and individuals with inapparent infections constitute a major problem in the control of the disease.

The clinical severity of a given case of bacillary dysentery is modified by relatively nonspecific factors such as the age and general condition of the patient, and the opportunities for supportive therapy. Some correlation exists between the species involved and the severity of clinical manifestations, as *Sh. dysenteriae* and, to a lesser extent, *Sh. ambigua* in general cause a more serious form of the disease than *Sh. paradyenteriae*, members of the para-Shiga group or *Sh. sonnei*. *Sh. alkalescens* is rarely associated with clinical dysentery while *Sh. dispar* is regarded as nonpathogenic, and hence of no clinical significance.

## IMMUNITY

The mechanism of spontaneous recovery from bacillary dysentery is not understood. Although humoral antibodies appear in response to infection, there is no evidence that they affect the recovery process directly, nor is there a correlation between their presence or absence and the occurrence of relapses and second attacks (Watt and DeCapito, 1945). As in other enteric diseases, there is no evidence that bacteriophage plays a decisive role. The local tissue response may be a factor; the exudate in bacillary dysentery is rich in polymorphonuclear leukocytes, and it is known that phagocytosis and destruction of bacterial cells may take place even in the absence of specific antibody.

Although relapses and second attacks of bacillary dysentery do occur, it is common experience in the tropics that individuals settled in areas where the disease is endemic tend to become immune to frank clinical



attacks of it. The nature of this clinical immunity is not clear, and careful bacteriologic and immunologic studies will be necessary to determine if it is species specific only, or perhaps broader, giving some protection against other members of the genus as well.

The injection of killed dysentery bacilli into human or animal subjects stimulates the production of antibodies which confer considerable protection upon mice against the intraperitoneal inoculation of the homologous organism. The relatively high toxicity of the vaccines have limited their use in man, but the results in controlled experiments in which human volunteers were first vaccinated and then challenged with the same type (given orally) have shown no evidence of significant protection against the infection (Shaugnessy et al., 1946). This experience seems reasonable when one remembers that the disease process is limited to the intestinal mucosa, and that there is no tendency to invade the blood stream. Under these conditions it is difficult to expect humoral antibodies to be effective. Antibacterial serum therapy has been equally disappointing, and the results of the treatment of infections due to *Sh. dysenteriae* with Shiga antitoxin are inconclusive.

### DIAGNOSIS

The laboratory diagnosis of bacillary dysentery is made most satisfactorily by the isolation of the specific organism from the rectal wall or from the feces of the patient. During the acute phase of the disease the organisms are excreted in large numbers and fresh stool cultures frequently give positive results, particularly if the sample contains mucus, as the bacteria are found in greatest numbers in this exudate (Boyd, 1940). Since dysentery bacilli tend to die out rapidly, the stool must be cultured as soon as possible. If delay is unavoidable a preservative such as 30 per cent neutral glycerine should be employed. The rectal

swab technic as first employed by Hardy et al. (1942) offers a better chance of isolating the organism since the culture is taken directly from the rectal wall, and streaked out on differential media at the bedside. Sigmoidoscopy with culturing of the actual ulcerations of the intestinal wall under direct observation (Ferris and Fortune, 1944) carries the process one step further.

Mucus or, failing this, fecal material, is emulsified in saline before streaking; if the swab is used, direct plating is carried out. At the same time the type of cellular exudate may be determined by examination of a stained smear; a predominance of polymorphonuclear cells suggests dysentery of bacillary origin, while a predominance of mononuclear cells favors a colitis of protozoal origin. As in the case of suspected *Salmonella* infections, selective media are used for plating: all these contain lactose, and an indicator such as neutral red which will detect any fermentation of lactose. Such media permit rapid differentiation between prompt lactose-fermenting organisms, such as the coliform bacilli, and late or non-lactose-fermenting bacteria such as the dysentery bacilli. It is best to use two media—one relatively noninhibitory, such as MacConkey's agar, and the other an inhibitory one, such as Shigella-Salmonella thiosulfate-citrate bile agar which largely suppresses the growth of coliform bacilli as well as that of Gram-positive organisms. Suspicious colonies are then fished into carbohydrate broths (lactose, glucose, mannitol, xylose, sucrose, salicin and dulcitol) for the detection of characteristic fermentation reactions, into peptone water for the determination of indole and hydrogen sulfide production, and inoculated into semisolid agar by stabbing for the detection of motility. In connection with fermentation reactions it must be remembered that *Sh. sonnei* is a late lactose fermenter; it rarely attacks this sugar before 48 hours, and usually later. Once an organism has been provisionally

grouped by means of these biochemical reactions its final identification should be carried out by the use of specific typing antisera, employing the method of slide agglutination.

Shiga (1898) was the first to show that the patient suffering from bacillary dysentery develops specific antibodies against the infecting organism. These are most conveniently demonstrated by means of the agglutination reaction. The serologic diagnosis of the disease is, however, not a very practical procedure. The multiplicity of species capable of causing the disease necessitates setting up the agglutination test against a number of bacterial suspensions. Individuals free of the clinical disease frequently have agglutinins against several *Shigella* species; since the clinical significance of these "natural" antibodies is uncertain, the interpretation of agglutinin titers obtained by testing a single specimen of serum is difficult. More convincing is the demonstration of a significant rise in titer against a specific organism during the course of the disease. This procedure requires the examination of at least two serum specimens and, since at best it furnishes the diagnosis in retrospect, its usefulness as a routine diagnostic tool is limited.

### SPECIFIC TREATMENT

Serotherapy has proven disappointing. The production of high-titer antitoxin against the so-called "exotoxin" of *Sh. dysenteriae* is practical, and, since infections caused by this organism are usually severe, the treatment of these cases with specific antitoxin has been recommended and practiced. Convincing proof as to its efficacy in modifying the course of the disease is lacking. Serotherapy has also been used in cases of *Sh. paradyserteriae* infection; because of the multiplicity of types within this species, it is necessary from the practical standpoint to use a polyvalent antiserum. Here

again the results have not justified its use, and today serotherapy is little practiced.

Bacteriophage therapy has received wide trial, and here, too, the results have been disappointing. In many clinical trials the lack of an adequate number of control cases has made the results difficult to evaluate, but on the whole they have been unconvincing. The carefully controlled experiments which Boyd and Portnoy (1944) carried out in North Africa during World War II yielded no evidence of therapeutic or prophylactic efficacy on the part of the bacteriophage preparations employed.

Chemotherapy holds more promise. Many of the sulfonamides exert a bacteriostatic effect upon organisms of the genus *Shigella* in vitro, and clinical trials have yielded favorable results. The relatively soluble, easily absorbed drugs have been shown to be efficacious even in small doses, and sulfadiazine and sulfathiazole are the drugs of choice today (War Dept., 1945). The dosage usually recommended is one gram four to six times daily. Drug-fast strains are met with, however, and these limit the usefulness of these agents.

Penicillin has been of no value. Streptomycin has given promising results in the relatively few cases in which it has been tried.

### EPIDEMIOLOGY

Bacillary dysentery is traditionally a disease of the tropics; it has, however, a world-wide distribution. With one exception, the more important pathogenic species are found in most areas of the globe; *Sh. paradyserteriae* and *Sh. sonnei* present a particularly wide distribution. The exception is *Sh. dysenteriae*, which in recent years has been largely limited to the Far East, India and the Near East (Weil, 1947).

The source of infection is essentially man. The *Shigella* are strict parasites, and no natural animal reservoirs have been detected. Their natural habitat is the gastrointestinal tract of man, and in cases of



clinical dysentery they are excreted in large numbers. As the patient recovers the organisms concomitantly tend to disappear from the stools. In some instances, however, organisms may persist during convalescence or even longer—and occasionally a carrier state develops. Subclinical infections are also responsible for some carriers, and since there are no signs or symptoms to attract one's attention to the infection these individuals may play an important role in the spread of the disease.

The spread from man to man may take place directly through the contamination of inanimate objects such as glassware, crockery, doorhandles, toilets, etc., which are used in common by many people, or indirectly through the medium of contaminated food or water supplies. Foodstuffs are usually infected by the dirty fingers of foodhandlers, and water through the medium of faulty plumbing, lack of protection of the source of supply, or inadequate chlorination (or other means of sterilization). In warm climates under primitive sanitary conditions (such as may accompany military operations, for example), flies play an important role in the spread of the disease (Taylor, 1919), and in some areas a direct correlation between the fly season and the onset of dysentery has been noted. In temperate climates and in areas where sanitary standards are high this method of spread is usually of minor importance. Although dysentery bacilli have occasionally been detected in the gut of flies, multiplication does not appear to take place to any extent, and transmission by these insects is a mechanical rather than a biologic process. They feed voraciously upon exposed fecal material which becomes adherent to their feet, antennae and proboscis. If subsequently they have the opportunity to walk over food or eating utensils, fecal matter containing dysentery bacilli may be deposited on them and by this means gain entrance to the body.

The transmission of bacillary dysentery

closely resembles the spread of other enteric diseases. During the acute illness the organisms are excreted in large numbers, and these through the medium of dirty fingers, faulty plumbing, or mechanical transmission by flies, are able to contaminate food and drink, and thus infect man once more. As compared to typhoid fever, there is a higher proportion of mild unrecognized cases but a smaller chance that the chronic carrier state will develop. In contrast to certain *Salmonella* species, dysentery bacilli show no tendency to invade the biliary tract.

### CONTROL MEASURES

The logical control of bacillary dysentery falls under four headings: (1) elimination of sources of infection, (2) prevention of spread, (3) increasing individual resistance, and (4) chemoprophylaxis.

Since man represents the only recognized host of the pathogenic *Shigella*, the elimination of these organisms from man should remove the great reservoir of the disease. Rigid precautions should be taken with the excreta of patients, and the latter should not be released from isolation until stool (or better yet, rectal-swab) cultures are negative on three or more successive days (Fortune and Ferris, 1945). The detection and isolation of mild subclinical cases and of symptomless or intermittent carriers represents an insuperable problem at the moment and limits the extent to which the elimination of the source of infection can be carried.

Since bacillary dysentery is spread largely through the medium of contaminated food and water, general sanitary measures aimed at better protection of water, milk, and food supplies, and at adequate sewage disposal and effective fly control are potent aids in preventing the disease. Large-scale outbreaks of bacillary dysentery are practically unknown in civilized countries. An exception to this is the so-called "asylum"

dysentery; both in the United States and in Europe bacillary dysentery is often endemic in large mental asylums and in orphanages where overcrowding and poor sanitary habits of youthful or mentally defective inmates give the disease many opportunities to spread. The introduction of a new strain under these conditions may initiate an explosive outbreak.

Attempts to increase the resistance of the individual against infection by means of active immunization have not been successful. The toxicity of vaccine preparations and the multiplicity of potential etiologic agents have handicapped clinical trials. Basically, however, it seems unlikely that the disease could be controlled by active immunization, for the absence of a septicemic phase, the brief incubation period and the localization of the pathologic changes in the gut wall make it far more analogous to the

acute gastro-enteritis caused by members of genus *Salmonella* than to typhoid or other enteric fevers.

The sulfonamide drugs have proven useful in the prevention of dysentery as well as in its treatment. Not only do chronic carriers frequently cease excreting dysentery bacilli after a therapeutic course of sulfadiazine but the administration of small doses, one or two grams daily, has been reported as being successful in checking outbreaks of the disease. Mass chemoprophylaxis may lend itself to the control of the disease among military personnel operating in the field under unsanitary conditions. Drug-fastness is encountered among the *Shigella*, however, and the presence and selective survival of drug-fast strains may render chemoprophylaxis ineffective in controlling an outbreak of bacillary dysentery (Cheever, 1946).

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# 19

## The Pasteurella

### INTRODUCTION

Under the tribe *Pasteurellae* (Castellani and Chalmers, 1919; Winslow et al., 1920), the Committee on Nomenclature of the Society of American Bacteriologists has grouped in the first genus, *Pasteurella*, those organisms apparently identical and producing similar diseases which Hueppe in 1886 had called *Bacterium septicemiae haemorrhagiae*. Trevisan (1887) proposed that the disease agents be recognized as separate species, but that they be grouped in a single genus to be named "Pasteurella," in honor of Pasteur. Sometime later (1896), "le bacille de la peste" (Yersin, 1894) or *Bacillus pestis* (Migula, 1900) was designated *Pasteurella pestis* by Lehman and Neumann and the Committee. Although culturally similar to the hemorrhagic septicemia organisms, this organism has different nutritional requirements and produces a different kind of disease. The bacillus isolated by Malassez and Vignal from a guinea pig in 1883 and described as *B. pseudotuberculosis* (Eisenberg, 1891) or *Streptobacillus pseudotuberculosis rodentium* (Preis, 1894), is related to the group immunologically and was added to the genus by the Committee, irrespective of the fact that it is motile. In 1932 Reiman and the Committee assigned *Bacterium tularense* (McCoy and Chapin, 1912) to the pasteurella group because it took a bipolar stain, was soluble in sodium ricinoleate and was vector transmitted. The extremely polymorphous character of the organism, its physiologic and biochemical characteristics, nutritional requirements and marked cytotropism have since made obsolete its inclusion in the genera *Pasteurella* or *Brucella* (Topley and Wilson, 1931). However, the designation *Bacterium tularense* is retained despite

its close relationship to the pleuropneumonia group.

### HEMORRHAGIC SEPTICEMIA PASTEURELLA MULTOCIDA

#### INTRODUCTION

These organisms are small, Gram-negative, ovoid rods showing bipolar staining by special methods; they include nonmotile, aerobic and facultative anaerobic bacillary forms which require a low oxidation reduction on primary isolation. Their powers of carbohydrate fermentation are relatively slight; they do not ferment lactose or produce gas; they produce indole, frequently  $H_2S$ , do not liquefy gelatin or coagulate milk, and require nicotinamide and pantothenic acid as accessory growth factors. They parasitize man, other mammals and birds in which they cause pasteurellosis or hemorrhagic septicemia.

For practical purposes *Pasteurella multocida* is indistinguishable from the avian, mammalian and human strains described as *Pasteurella avicida* or *Pasteurella aviseptica* (Kitt), *Pasteurella bollingeri* or *Pasteurella suilla* or *Pasteurella suiseptica*, *Pasteurella muricida* or *Pasteurella muriseptica* and *Pasteurella cuniculicida* or *Pasteurella lepiseptica* (Fig. 2N).

#### HISTORY

Discovered at the dawn of bacteriology, these organisms were used extensively in many of the early bacteriologic and immunologic studies. It was found that fowl cholera, rabbit septicemia, Wildseuche (a



fatal disease of deer) and swine plague were caused by small bacilli which, because they stained deeply at the poles, were designated *Bacillus bipolaris septicus*. The bacteriologic position of the hemorrhagic septicemia group was established on a broader basis by Lignières (1900), but present-day knowledge invalidates his zoological grouping of the pasteurilla according to animal origin of each individual strain. According to Schütze (1929) 230 strains isolated from reindeer, cattle, buffaloes, sheep, pigs, cats, chickens, rabbits and rats and described by 17 authors revealed great similarity; all were immotile, none fermented lactose. Bacteriologic characteristics and similarity of pathologic lesions in various animals make it likely that all typical pasteurilla belong to a single species. Rosenbusch and Merchant (1939) proposed the name *Pasteurella multocida* Kitt, for all typical indole producing, nonhemolytic hemorrhagic septicemia organisms. The name indicates that the organism has more than one host. The innumerable strains composing the group fluctuate easily and continuously in physiologic functions; antigenic structure, fermentation capacity and pathogenic ability. Moore (1895) demonstrated that the pasteurilla are saprophytic in the respiratory tracts of a great many animal species. The first bacteriologically proved human *Pasteurella* infection was reported in 1913 by Brugnattelli.

#### MORPHOLOGY

*P. multocida* vary from small, short oval forms to coccobacilli with convex sides and rounded ends. They range in length from 0.3 to 1.25  $\mu$ , with diameters of from 0.15 to 0.25  $\mu$ , and appear singly or in pairs, small chains or clusters. Active healthy specimens from cultures stain easily and diffusely with aniline dyes and are Gram negative, but in animal tissues and fluids the two ends of the rod stain more intensely than the central

portion. Pleomorphism is not common, although filamentous, granular or barred bacillary forms without bipolar staining are seen in preparations made from rough colonies. Spores are not formed and the organisms are nonmotile and without flagella. *P. multocida* is not usually regarded as an encapsulated organism, but Priestley (1936) demonstrated envelopelike structures in cultures of a virulent strain grown at 37° C., as did Regamey (1938) in the leukocytes of tissue smears. That a capsule-like structure does exist is indicated by the viscosity of cultures and animal exudates.

#### CULTIVATION AND BIOCHEMICAL ACTIVITIES

Moderate growth occurs on ordinary media at 37° C. Under aerobic conditions, *P. multocida* will develop through 2 or 3 transfers in hydrolyzed gelatin or amino-acid basal media with nicotinamide (phosphopyridine nucleotides), pantothenic acid and biotin concentrates (Berkman, 1942). Traces of rabbit blood or oxygen pressure favor growth of the D variant of *P. lepisepticum*. Colonies grown on weakly alkaline agar (pH 7.2 to 7.6) at 37° C. are small, fine, dewdrop-like, translucent; frequently they appear to be fluorescent or blue-whitish and hyaline. When several days old, they may be viscid and adhere to the media. Growth is more luxuriant on blood agar, coagulated serum or egg media. Colony variants in cultures of *P. lepisepticum* have been described by de Kruif (1921): Type D grew profusely in broth, formed fluorescent opaque colonies and was highly virulent for rabbits, while Type G gave a granular deposit in broth, formed translucent bluish colonies with little fluorescence and was completely avirulent; Type D gave rise to G variants but G never reverted to D. Webster described a mucoid variant of intermediate virulence. Similar variants have been reported for *P. aviseptica* (Hughes, 1930).

*P. multocida*, as a rule, causes uniform turbidity in broth, which in 4 to 6 days becomes clear with a viscous sediment. Gelatin is not liquefied, milk remains unaltered, and no visible growth takes place on potatoes or on MacConkey's medium containing bile salts. Cultures may have a characteristic odor.

In general, *P. multocida* produces acid in glucose, saccharose, levulose, sorbitol, galactose, mannose, xylose, trehalose and mannitol, with some exceptions in the last three media. No acid is formed in dulcitol, raffinose, rhamnose, adonitol, dextrin, inulin, glycerol or erythritol. Some avian strains are acidogenic in maltose, while buffalo, reindeer, calf and rabbit strains occasionally ferment lactose (Tanaka, 1926). On the basis of fermentation of xylose or arabinose, or both, Rosenbusch and Merchant divided strains of *P. multocida* into three subgroups, each of which showed a considerable degree of antigenic homogeneity. Peptone-water cultures with 0.5 per cent glucose may reach pH 5.6-6.1 (Otten, 1926). All members reduce nitrates and form ammonia, indole and  $H_2S$  in varying small amounts; they give a positive catalase test but a negative methyl red reaction; many reduce methylene blue. The true pasteurella form no hemolysin, but reduce oxyhemoglobin to hemoglobin. Jones (1921), Newsom and Cross (1932), Beveridge (1937) and others have described strains producing beta-hemolysin on rabbit or horse blood agar. These strains fail to produce indole, ferment maltose, lactose and inositol and are nonpathogenic for mice and rabbits. Whether or not they form true hemolysins has not been determined and the creation of a so-called hemolytic subgroup within the hemorrhagic septicemia group may be premature.

Drying, sunlight, heating above 50° C., 0.5 per cent phenol or 0.1 per cent formalin easily destroy *P. multocida* organisms in 15 minutes. Cultures mixed with unsterilized mud and protected from sunlight remained alive for at least 3 months (Holmes, 1914). *P. multocida* are sensitive to 0.2 units or less of penicillin per cc. (Schipper, 1947). Virulent stock cultures can be preserved on blood agar in sealed tubes.

#### ANTIGENIC STRUCTURE

Antigenic relationships between all members of the subdivision are close. Agglutinating serum prepared against any one of the pasteurella reacts with the homologous strains, and to a lesser extent with heterologous strains (Rosenbusch and Merchant, Schipper). The antigenic structure is still uncertain, although observations by Priestley (1936), Dingle (1934) and Pirotsky (1938a, b) suggest that *P. multocida* form envelope and somatic antigens. Using the technic of Boivin with trichloroacetic acid, Pirotsky isolated four different glycolipoid antigens, one of which, by cross-precipitation tests, appeared to be related to the Vi antigen of the typhoid bacillus, while another reacted with the O antigen of salmonella. However, the relationship of the glycolipoid antigens to the envelope or capsular antigen has not yet been determined.

Ever since Kitt protected fowl against fowl cholera with the bacilli of rabbit pasteurellosis, many investigators have proved such a reciprocity between all animal strains of pasteurella. Although the serum from a horse, cow or rabbit hyperimmunized with a strain of pasteurella can protect against hemorrhagic septicemia infection with another species, protection sometimes requires the use of homologous sera (Shirop, 1908). Old cultures (Hadley, 1918) and the bodies of *P. bovisseptica* and *P. suisseptica* (MacFadyen, 1907) yield soluble toxic substances which bring about in laboratory animals stupor, diarrhea, edema and death, toxic effects probably due to endotoxins liberated during autolysis.

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

Pasteurella are saprophytic and parasitic in some members of the animal kingdom in nearly every country of the world. In acute fatal infections they may be isolated from



blood, organs and exudates. In chronic infections, their precise relationship to various pulmonary lesions and abscesses in which they are seen is not always entirely clear. Pasteurella are found very frequently in the upper air passages of normal cattle, horses, swine, sheep, fowl, dogs, cats and rats (Schipper, 1947). Less frequently they are normal inhabitants of the intestinal tracts of these animals (Pritchett et al., 1930). A more or less saprophytic strain may acquire marked virulence, through passage, and then may suddenly assume again its saprophytic nature. All pasteurella are highly and more or less specifically pathogenic. The total range of susceptible animal species is wide, including man, rodents, herbivores, fowls and possibly carnivores, but each host has its characteristic limitations beyond which it rarely goes in spontaneous disease. The range of pathogenicity for each member of the group, so far as known, is given below.

Strains of avian origin induce septicemia in rabbits, mice and field mice, while guinea pigs, cattle, horses and sheep develop swellings or abscesses at sites of subcutaneous injections. Pasteurella isolated from cattle, buffaloes, reindeer, camels, elephants and sheep are highly virulent for rabbits, and less so for mice, guinea pigs and pigeons; chickens and horses are quite resistant. Pasteurella of porcine origin infect guinea pigs, mice, rabbits and fowl. Guinea pigs spontaneously infected with pasteurellosis transmit the agent aeri-ally to mice (Freund, 1926). Strains isolated from Norway rats by Meyer and Batchelder infected guinea pigs, rabbits, laboratory and wild rats but not barnyard fowl or cats. Exposure of sick wild rats to white rats incited a small epidemic. Schipper (1947) found saprophytic strains isolated from the nasopharynx of Norway rats to be highly pathogenic for guinea pigs, hamsters, cotton rats, alexandrinus rats, chickens, cats and dogs, but rats of the Norway, hooded and albino types even resisted intraperitoneal injections. Pasteurella of mouse origin are virulent for rabbits and guinea pigs but not for pigeons. Feline strains are lethal for rabbits, guinea pigs and mice. Human strains of *P. multocida* are pathogenic for mice, rabbits and occasionally pigeons;

they produce local abscesses on subcutaneous injection in guinea pigs.

#### PATHOGENESIS

The equilibrium between the virulence of a given *P. multocida* strain and the susceptibility of a given host governs the resultant pathologic process. If the bacilli are highly virulent and the resistance of the animal slight, the clinical picture of acute or peracute septicemia is produced; bacilli multiply rapidly in the tissues, causing death within 12 to 36 hours. There is high fever, cardiac weakness, prostration, toxemia, anorexia and sometimes diarrhea. Autopsy findings, aside from enormous numbers of bacilli in the blood, may be essentially negative or there may be blood-tinged effusions in the serous and mucous membranes and in the parenchyma of organs. The spleen is not enlarged, but the lymph nodes are swollen. When the disease lasts for several days localized inflammatory and necrotizing processes induce varied clinical pictures. Pathologically, there are hemorrhagic serous infiltrations, fibrinous pneumonia with localized caseating, necrotic foci of varied sizes, serofibrinous exudation of the pleurae, swelling of the lymph nodes and sometimes inflammation of the joints and tendon sheaths or even of the udder (Lowell, 1939). In fowl cholera, focal pin-point necroses in the liver, cheesy exudate in the peritoneum and petechiae on the pericardium are common. The portal of entry in natural infections seems to be the gastro-intestinal tract, through ingestion of contaminated food and water, although the studies of Pritchett, Beaudette and Hughes (1930) indicate that fowl are more readily infected by the respiratory route. Pasteurella sometimes play an important secondary role in certain virus diseases such as swine fever, distemper, or equine pleuropneumonia by invading respiratory mucous membranes earlier damaged by a virus. Spontaneous hemorrhagic septicemia of rats (Meyer and

Batchelder) and mice (Greenwood et al., 1926) has all the characteristics of a respiratory infection. *P. lepi-septicum*, quite commonly found in nasal secretions of rabbits, may cause local infection such as snuffles, paranasitis, otitis media, subcutaneous abscesses, pleuropneumonia, and septicemia when seasonal changes or the injection of harmful substances have lowered resistance against disease. Infection normally occurs through the respiratory tract and proceeds by process of extension.

#### IMMUNITY

Individual variations from extreme susceptibility to complete resistance, the ways in which that resistance is influenced by hereditary or acquired immunity, and seasonal or other environmental factors apparently control the immunity mechanism. Although the illuminating observations of Pasteur on the loss of virulence of fowl cholera organisms, and on the immunizing value of avirulent cultures have been of fundamental importance in the study of immunity, practical applications to pasteurella infections are still limited. Many difficulties have been experienced with active immunization by inoculation of fowl with living avirulent cultures. There is evidence from the work of Hadley and Aimson, of Manninger and of Anderson et al. (1929) that colonial variants of certain avian strains differ enormously in immunizing properties. Two injections of a suitably avirulent strain give solid though temporary immunity against virulent cultures (Nobrega and Reis, 1938). As Kitt and others have demonstrated, strains causing rabbit pasteurellosis or pleuropneumonia of calves may serve as vaccines against fox cholera. Living avirulent cultures have been used to protect sheep and swine but are considered too dangerous for practical field investigations since the bacilli persist in the tissues and are likely to spread the disease.

Killed antigens apparently do not produce a sufficiently protective immunity. Weil (1908) applied to fowl cholera Bail's aggressin principle, and for a time aggressins prepared from exudates or agar cultures killed with phenol or heat were extensively used. Subsequently Harvey and Iyengar (1925) and Sakamoto (1922) achieved notable results in pigeons by using killed homologous or heterologous antigens, but evidence of definite protection was by no means convincing. Moreover, it is necessary in estimating the value of protective inoculation against pasteurellosis to realize that the disease tends to subside spontaneously. It is difficult to form an idea from the numerous reports as to the real prophylactic value of vaccination against porcine, bovine and ovine pasteurelloses. The work of d'Herelle and Le Louet (1926), who claimed to have immunized buffaloes against epizootic bovisseptica by inoculating them with bacteriophage isolated from dejecta of healthy animals, has not been confirmed. Of interest are the observations of Hadley (1924) that rabbits are readily immunized with antigens, provided large, repeated inoculations are given. The method used for killing the antigen, whether by heat, chemicals, dyes or by sonic vibrations, has little influence on the immune response, provided the inactivated material is injected intravenously (Langner et al., 1941).

Passive protection has been achieved with antisera derived from immunized animals, including horses and cows. Although their protective action is transitory and of no great value in treatment, their effectiveness in staving off death makes them useful in combined serovaccination; they have been so used in fowl cholera, swine plague and pneumo-enteritis of sheep. Sera of immunized animals continue to be employed to reduce mortality due to pasteurellosis in poultry flocks and herds of cattle as well as in buffaloes in India and Indo-China (Bennett, 1926; Jacotet, 1940).



## DIAGNOSIS

Pasteurella infection cannot be diagnosed with certainty in living animals. Moreover, the anatomic changes do not always permit an etiologic interpretation without preliminary bacteriologic examinations. Microscopic cultural and biochemical examinations followed by pathogenicity tests on mice, guinea pigs, rabbits and pigeons and supplemented by serologic tests with typing sera will readily differentiate *P. multocida* from *P. pestis* and *P. pseudotuberculosis rodentium*.

Topacio (1939) has emphasized the difficulty of diagnosing pasteurellosis in buffaloes by microscopic and cultural methods; he advises that rabbits, guinea pigs, rats and mice be inoculated whenever the worker fails to detect the agent in smears from blood and spleen. In the more chronic forms of pasteurella infection the organism is not infrequently associated with other micro-organisms, for example, *B. bronchisepticus* in snuffles (Webster et al., 1927). Shook and Bunyea (1939) used a rapid whole-blood test with an antigen containing 0.01 per cent crystal violet to detect carriers in poultry flocks. Nasal washings may be inoculated into mice to detect Pasteurella in the paranasal sinuses of rabbits and rats.

## CHEMOTHERAPY

The success of sulfonamides in the treatment and prophylaxis of plague gives promise that these drugs may be useful in the pasteurelloses.

A small clinical experiment by Shanks (1941) on a flock of turkeys suffering from fowl cholera suggests that sulfapyridine may be of value. On the other hand, Boschnakoff (1941) found that "streptosil" had no effect on *P. equiseptica* and Cooper and Moore (1945), treating a local pasteurella infection of the hand with sulfonamide gauze, noted no beneficial effects. Queen and Quortrup (1946) found a strain of pasteurella recovered from wild ducks sensitive to 0.08 units per cc. penicillin in vitro; the antibiotic protected wild ducks infected experimentally with this strain. Schipper (1947) reports that all pasteurella

obtained from the throats of Norway rats proved sensitive to 0.2 units of penicillin per cc. or less.

## EPIDEMIOLOGY

The magnitude and economic significance of herd and flock losses due to pasteurelloses are in part reflected by recent data from Burma. During the year 1939-1940 4,293 cattle died from hemorrhagic septicemia and 51,683 vaccinations were practiced. The epidemiology of pasteurella infections is not altogether clear. Hueppe's original view, which regarded the soil as the medium of growth and source of infection, is still shared by some observers (Gomez, 1926). Pasteurella are widely distributed in the respiratory and intestinal tracts of healthy mammals and birds. Matzke, for example, recorded a carrier population of 3.3 per cent in a flock of chickens; Schipper found 12 per cent of a group of 102 Norway rats to harbor pasteurella in the nasopharynx; and according to Schenk (1938) 15 of 20 cats were similarly infected. The carrier rate may vary from 35 to 60 per cent on a rabbit farm where the disease is entrenched and chronic. It is often difficult or impossible to determine how fowl cholera is introduced, but Pritchett et al. (1930) in a study of several flocks in New Jersey give some evidence that carrier birds may be the initiators. They found that explosive outbreaks ensued when healthy birds had contact with carriers. On the other hand, it is claimed by others that the incidence of fowl cholera is no greater in flocks having chronic carriers than in those without carriers. Schipper (1947) postulates that the wild rat, serving as a reservoir of *P. multocida*, may be the missing link in the puzzling epidemiology of fowl cholera. And it is established that in severe epidemics of this disease even highly virulent pasteurella survive with difficulty in the host, whereas the endemic strain is relatively low in virulence and is highly vegetative.

Preceding an outbreak of snuffles and pneumonia in rabbits the carrier rate invariably rises. In cattle and buffaloes there is evidence that epizootics may develop when carrier resistance is weakened by environmental factors, and the virulence of the parasite is at the same time enhanced. Observations in Cochin-China correlate the incidence of hemorrhagic septicemia in buffaloes, cattle and pigs with heightened humidity and rainfall which reduce the body temperatures of the animals by 1° to 2° C. European statistics, particularly from Rumania, clearly show a connection between cold weather, vitamin-A deficiency and incidence of fowl cholera; the disease does not appear in well-nourished chickens. Further, it is suspected that when the respiratory passages of cattle are infected with *Dictyocaulus viviparus*, the animals may be less resistant to pasteurella. Magnussen suspected insect spread in large epidemics among reindeer; and Daubney et al. (1934) found the flea *Ctenocephalus felis* to carry pasteurella. Although Skidmore (1932) and Nieschulz (1939) mechanically transmitted fowl cholera with the flies *Musca domestica* and *Tabanus*, infection hazards from flies are not nearly so great as from carrier fowl in the flock itself.

Human infections with *P. multocida* are being more frequently recognized (Lévy-Bruhl, 1938; Weber, 1941, Schipper, 1947). In 55 cases (with 5 deaths) not always proved according to modern methods of bacteriology, the infection was local with and without generalizing symptoms. In almost half, animal bites were precursors of infection: 21 cat bites, 4 dog bites, 1 rabbit bite and 1 panther bite were proved responsible. Exposure to cattle, pigs or rabbit carcasses (muscle particularly) or consumption of infected rabbits brought on enteritis, conjunctivitis, appendicular abscesses and other manifestations. The generalized infections, symptoms of which vary widely from protracted, recurrent chills and fever to pneumonia and empyema (12 cases), meningitis (7 cases), and puerperal sepsis have been reported mainly from Europe. The sources of infection remained undetermined. Generalized infections have frequently proved fatal. Cases of meningitis associated with pasteurella, of which 7 are

recorded, are usually sequels to skull fractures or sinusitis. It is not unlikely that the offending organism which induces meningitis following skull fracture comes from the nasal passages. Topley and Wilson observed the persistent carrying of a pasteurella in the nose of an animal caretaker. Particularly interesting is the fact that *P. multocida* apparently may lie dormant in the tissues for months and only after traumatization of subacutely infected tissues cause acute infection. Localized human infections, which may be limited to phlegmon and abscess, are only too frequently complicated by osteomyelitis (Allott et al., 1944; Hausmann and Tully, 1945). In the city of Munich, where many cat-bite wounds were noted (Weber, 1941), 75 per cent of the cats harbored pasteurella in their nasal passages. Although the sera of patients tested for subacute infections in a few instances agglutinated the pasteurella isolated from the disease process in relatively high dilutions, the majority of the tests were negative.

#### CONTROL MEASURES

Proper feeding and sanitary management of chicken flocks and hog ranches are more effective and economical than treatment or biologic prophylaxis. In countries where civilization is primitive and sanitation all but nonexistent, vaccination of cattle, buffaloes and hogs still has its advocates.

### PLAGUE PASTEURELLA PESTIS

#### INTRODUCTION

The plague bacilli are large, Gram-negative, elongated rods with rounded ends, which exhibit very marked bipolar staining in the parasitic stage; coccus-like, round, filamentous or other pleomorphic forms are common (Figs. 1P and 1Q, Fig. 2P). The immotile organism grows on media containing bile salts, has only slight powers of carbohydrate fermentation, is



unable to ferment saccharose, produces neither indole nor sulfured hydrogen, is milk neutral and requires no accessory growth factors.

*P. pestis* causes a disease primarily affecting wild rodents and rats which is maintained as a continuous heterogenous infection chain through an insect vector, the flea. Man becomes a victim of the bubonic form accidentally by interpolation in the rat-flea-rat sequence or by handling infected wild rodents. Should the acute, febrile, highly fatal human disease lead to the pneumonic form, man-to-man infection ensues without intervention by the flea. [Synonyms: *Bacille de la peste* (Yersin, 1894), *Pest bacillus* (Aoyama, 1895), *Bacterium pestis* (Lehman and Neumann, 1896), *Bacillus pestis* (Migula, 1900), *Yersinia pestis* (van Loghem, 1944-1945).]

### HISTORY

The history of plague can be traced back almost uninterruptedly to the third century before the Christian era when Dionysius told of it as a fatal disease in Libya, Egypt and Syria. The various pandemics and epidemics of history have been examined and interpreted in the works of Simpson (1905), G. Stricker (1908 and 1910) and Wu Lien-Teh et al. (1936). For centuries the "Black Death" or "Great Mortality" found in Europe a highly susceptible population living in poverty and took an appalling toll, justifying all of its somber aliases and the awe in which it was held. After the well-known outbreaks in Milan (1630), London (1665) and Marseille (1721), sanitary standards, better housing and legalized prophylactic measures led to retrogression of the disease; epidemic plague had disappeared from Europe by 1843 and was no longer to be found in Asia Minor, Syria or Palestine (Meyer, 1947). The modern pandemic originated in Yunnan on the Tibetan border of China, reached Canton early in 1894, from there was carried by steamer to Hong Kong and thence to Bombay within the year; the next 20 years saw India become the scene of an appalling epidemic. Since vessels moved in and out of Hong Kong unrestrictedly, it became the focus of infection from

which plague was disseminated to every important country of the world. It was in Hong Kong that the causal organism (*Pasteurella pestis*) was discovered by a Swiss, Alexander Yersin, on June 20, 1894 (Noel Bernard, 1944). His name is often coupled with that of Kitasato, but on insufficient grounds. It is true that on July 7, 1894, Kitasato announced the finding of an organism in plague cases, but he erroneously described it as Gram-positive and slightly motile (Lagrange, 1926; Severn, 1927).

After Yersin's discovery of the bacillus India became the most fruitful center of plague work, and as the epidemics there increased in severity, various European governments sent commissions to Bombay. Their investigations, published in a series of excellent reports, did a great deal to further knowledge of the disease. Various workers, in comparing historical records of epidemics from many countries, had noted that rats began to die before human beings were affected, and that rodent and human outbreaks were apparently intimately connected. Ogata in 1897 suggested tentatively that the flea might play a part in transmission, but it was Paul Louis Simond, working in Bombay, who set down the main facts about the transmission, epidemiology and control of plague (Lowe, 1942; Noel Bernard, 1947). His hypotheses played an important role in the success of the anti-plague experiment in San Francisco in 1903, when the U.S. Public Health Service put them to test. The actual part played by the rat was made clear in the reports of the Plague Research Commission (1906-1917) which showed that contact with rats was not followed by plague if fleas were excluded, that healthy rats could be kept in contact with live infected rats and with rats dead of plague without contracting the infection, and that the disease was not acquired by ingestion. Plague-infected rats have been captured in epidemics the world over since the Canton outbreak and have been an important link in the transmission chain in

most of them. Rat plague was also demonstrated on many ships from 1896 to 1936. That rats are not the only animals to suffer from the disease became apparent when marmotlike animals, the tarabagans (*Arctomys bobac*), were found infected in Transbaikalia and Mongolia in 1895, plague-ridden squirrels (*Sciurus pulmarum*) appeared in India in 1898 and the South African striped mouse (*Rhabdomys pumilio*) was found infected in 1906. Today the number of rodents definitely known to harbor spontaneous or "sylvatic" plague is great, and the transition from wild to domestic species has attracted attention not only in Russia, South Africa and the United States, but on the Peruvian-Ecuadorian frontier (Macchiavello, 1946) and in the Argentine as well (Meyer, 1942).

The mechanics of plague transmission from rats to man and among rats themselves was studied by Ogata (1897) who injected crushed fleas from an infected rat into mice and produced disease. The English Plague Commission working in Bombay (1905-1906) established on definite experimental evidence that the flea *Pulex* (*Xenopsylla cheopis* of Rothschild, 1903) transmits plague from rat to rat. How the bacilli multiply in the stomach of the flea and are then regurgitated during the sucking act of the insect was worked out by Bacot and Martin (1914). More recently, important contributions to the study of fleas involved in sylvatic plague have been made by Eskey and Haas (1940), Wheeler and Douglas (1945) and Burroughs (1947).

Prophylactic immunization with killed antigens, initiated by Haffkine (1896-1897), has been improved by Sokhey (1936-1946). Vaccination with live avirulent cultures was introduced by Otten (1940), and has been studied by Girard (1934) and Grasset (1947). Anti plague serum has been used in therapy since the time of Yersin et al. (1895); more potent preparations were produced by Naidu, Mackie and Brist in 1931.

Sulfonamides, originally tried by Carman (1938), proved successful in field tests by Wagle et al. (1941). Lately, the prophylactic value of sulfadiazine has been proved experimentally, and the high therapeutic activity of streptomycin in experimental pneumonic plague has been established (Meyer and Quan, 1947).

#### MORPHOLOGY

*P. pestis* appear in the animal body as short, round coccoid or large ovoid, safety pin-shaped bacilli with a length of from 1.5 to 2.0  $\mu$  and a width of from 0.5 to 0.8  $\mu$ . They are Gram negative and the typical bipolar staining of single organisms or pairs contrasts with the uniform color of the spherical bodies. In cultures bipolar staining is usually not so well seen, and on artificial media, particularly liquid, the formation of chainlets and larger rods is common. The stain usually recommended for plague work is 1 per cent carbolthionin, Wayson's dye mixture (methylene blue with carbol-fuchsin), or any modification of Romanovsky's or Giemsa's solutions. Peculiar mold-like and yeastlike serpentine formations and feebly staining irregular bladder and ring types are involution forms which appear in environments unsuitable for the growth of the bacilli, viz., decomposed carcasses of infected rats or 3 per cent sodium chloride in agar. True capsules with definite edges form irregularly in vivo, while in media containing heated sera at 37° C. the bodies of the rods are surrounded by ill-defined "envelopes" demonstrable in India-ink preparations. Highly virulent strains possess envelopes about as wide as the bacillary bodies (Rowlands, 1914; Bhatnagar, 1940). Certain avirulent strains give no microscopic evidence of envelopes. Electron micrographs clearly show the existence of both capsules and cell sap vacuoles (Marton and Meyer, 1945). *P. pestis* do not possess flagella and are immotile.



## CULTIVATION

On neutral or weakly alkaline media at an optimum temperature of 30° C. with a free oxygen supply, the plague bacillus grows slowly; colonies may not become visible before 24 hours. On moist agar the colonies are of slimy, viscous consistency. Bhatnagar (1940) describes numerous colony types, every one of which could be produced by all the strains he examined. Small smooth convex "dewdrop-like," sticky, colorless colonies with fringes predominate in virulent cultures, while larger colonies of a granular, rugose, yellowish, brittle and non-viscous nature frequently denote avirulence. Jawetz and Meyer (1943) confirmed the existence of such colony types, but found that variations in colonial character depend far more on minute differences in agar thickness and moisture than on inherent strain differences. Grown in broth, the smooth colony type produces uniform turbidity; the growth of the brittle type accumulates as a coarse, granular sediment with a completely clear supernatant. The well-known stalactite and stalagmite formations appear in broth tubes if sterile oil is floated on the surface. Predominantly smooth cultures recently made in vivo may not form stalagmites readily. *P. pestis* growth appears as feathery projections from the stab in gelatin cultures, without liquefaction. Potato is unsuitable. Special media have been developed which contain bile salts and gentian violet and are highly recommended for the isolation of *P. pestis* from contaminated tissues (Meyer and Batchelder, 1926).

Doudoroff (1943) obtained growth in a medium consisting of 0.2 per cent glucose, 0.1 per cent  $\text{NH}_4\text{Cl}$ , 0.05 per cent  $\text{MgSO}_4 + 7\text{H}_2\text{O}$ , 0.005 per cent  $\text{FeCl}_3$ , 0.001 per cent  $\text{CaCl}_2$  and M/30 Sorensen phosphates, buffer at pH 7.0, adequately aerated, provided small amounts (0.002 per cent) of cystine, phenylalanine, thiosulfate, sulfite or thioglycolate were added. Neither tyrosine

nor tryptophan appeared so satisfactory as phenylalanine. In the presence of cystine, mannitol was almost as good a carbon source as glucose. Amino acids are no more satisfactory than ammonium salts, and glycine does not possess the growth-promoting properties claimed by Rao (1939). Heavy cultures are obtained in synthetic media containing glucose, cystine and phenylalanine if adequate aeration is provided by constant agitation. Avirulent strains will develop under anaerobic conditions in complex media containing glucose. Wright (1934) found plague bacilli sensitive to oxygen, and surface cultivation improved when either 0.1 per cent blood or 0.05 per cent sodium sulfite was added to the agar. The organism grows in nutrient broth between -2° and +45° C.; at 27° to 28° C. growth is 5 times as rapid as at 37° C. (Sokhey and Habbu, 1943). Casein or gelatin hydrolysate media (Berkman, 1942) are being used in the mass cultivation of *P. pestis* for vaccines without the addition of accessory growth factors (Sokhey, 1945). The medium of choice for diagnosis and routine plague work is a 1 to 5 per cent rabbit-blood agar.

## BIOCHEMICAL ACTIVITIES

In the course of growth, broth cultures become alkaline and may reach pH 8.0 at the end of 9 weeks (Naidu and Jung, 1927); in peptone water with 0.05 per cent glucose the final pH is 4.6 to 4.9. No hemolysis occurs on blood agar plates, but there is slight browning. Glucose, levulose, maltose, mannitol and galactose are fermented with the production of acid but not of gas; lactose, saccharose, raffinose, dulcitol and inulin undergo no change. Aberrant reactions due to the use of unsuitable media such as peptone or serum water have been noted. According to Kurauchi (1937), all strains isolated in Manchuria and Russia, and some from South Africa, but none from China, India or Japan and none of 23 recently cultured strains from California are glycerin fermenters.

The chief products of glucose fermentation appear to be ethyl alcohol, carbon dioxide, and various acids—lactic, acetic, formic and succinic acids, and a small amount of pyruvic acid. It is generally agreed that the plague bacillus does not produce indole and causes no change in milk. It slowly reduces methylene blue and Janus green.

#### RESISTANCE

Sunlight sterilizes the plague bacillus in 3 or 4 hours; it is inactivated by heating at 55° C. for 15 minutes, by 0.5 per cent phenol in the same time, or by 95 per cent alcohol in 10 minutes. The majority of bacilli succumb during lyophilization. They remain viable in dried sputum for at least 3 months, and in dry flea feces held at room temperature for 5 weeks (Eskey and Haas, 1940). *P. pestis* is not sensitive to cold; cultures and organs held in the icebox retain their virulence for months or even up to 10 years. Virulent strains can be preserved by cultivating on 5 per cent rabbit agar at room temperature (26° to 32° C.) for 4 days, the tubes sealed and the material stored at 4° ± 2° C. (Sokhey, 1930). Some strains of *P. pestis* are killed by 0.02 units of penicillin, while by comparison 4 strains of *P. pseudotuberculosis* isolated from different animal hosts resisted as much as 1.5 units of the antibiotic. Streptomycin in concentration of 0.2 microgram/cc. is lethal to a recently isolated human strain; amounts varying from 0.4 to 4.0 micrograms/cc. of dihydrostreptomycin proved bactericidal to 6 plague strains in 5 days.

#### ANTIGENIC STRUCTURE

Wats et al. (1939) confirmed previous observations by Pirie that simple agglutination and agglutinin absorption tests fail to reveal the existence of types or groups within the species, or differences between avirulent and virulent strains. Schütze demonstrated that the plague bacillus possesses

two antigens: one contained in the gelatinous envelope, the other in the somatic portion of the bacterium. The envelope antigen is developed best at 37° C. and is heat labile, while the heat-stabile somatic antigen develops as well at 20° C. as at 37° C. These antigenic differences are reflected in the characteristic agglutination of the 37° C. and room-temperature cultures. Baker et al. (1947), by extracting acetone-killed virulent plague bacilli with neutral salt (0.85 to 2.5 per cent) or sodium acetate solution, obtained a water-soluble and a water-insoluble antigenic component. The water-soluble fraction is toxic for mice and rats (LD<sub>50</sub> 8 to 15 micrograms), and is highly immunogenic for these species. It contains at least 3 antigenic components: (1) a carbohydrate protein (Fraction 1A), soluble at 0.25 saturation of ammonium sulfate at pH 7.0 to 7.5 (Molisch positive); (2) a carbohydrate-free protein (Fraction 1B) soluble at 0.3 saturation of ammonium sulfate, and which crystallizes in the form of fine needles when the concentration of ammonium sulfate is raised to 0.33 saturation (Molisch negative); and (3) a toxic fraction soluble at 0.33 saturation of ammonium sulfate. Fractions 1A and 1B are similar immunologically. They produce potent antisera and absorb all of the antibody. Both induce immunity in mice but neither is of value in producing it in guinea pigs. Fraction 2, freed from Fraction 1 by absorption with Fraction 1A antiserum, has an LD<sub>50</sub> of 0.6 mg. for 20 gram mice. It produces antiserum which neutralizes plague toxin. The water-insoluble fraction or "residue" contains phenol-soluble and phenol-insoluble fractions; the antigen which protects guinea pigs is found in both fractions.

Serologic studies have shown that Fraction 1 is formed by virulent and certain avirulent strains which produce these antigens at 37° C., and form stabile salt suspensions. Avirulent strains or those grown at 26° C. produce only traces of Fraction 1. Antisera prepared with the surface antigen



Fraction 1 agglutinate all plague bacilli with envelopes in woolly, flaky aggregates, the so-called "envelope" type of agglutination. Uncapsulated strains or those with little envelope are agglutinated by antisera prepared against whole plague bacilli; the aggregates are fine, hard granules of the so-called "somatic" types. Such sera also agglutinate *P. pseudotuberculosis* as they contain antibodies to the common somatic antigens, but sera prepared against *P. pseudotuberculosis* will not agglutinate plague bacilli grown at 37° C., because the somatic antigens are protected by the envelope Fraction 1 antigens (Bhatnagar, 1940). The discovery of host specificity for plague antigens is of the greatest importance in explaining the immunogenic properties of antigens and vaccines used in the prophylaxis of the disease. Fraction 1 and consequently plague strains with envelopes are highly immunogenic for mice, rats, monkeys and apparently man, while the insoluble residues—the strains devoid of envelope antigen (No. 14 of Jawetz and Meyer)—immunize guinea pigs but not mice. Extracts from both virulent and avirulent plague strains contain a factor which enhances spreading and capillary permeability of the organisms.

#### TOXIN

A fraction highly toxic for mice and rats, less so for rabbits, guinea pigs and monkeys, and probably identical with the broth toxin first recognized by Markel (1898) is obtained by extracting acetone-killed dried *P. pestis* with physiologic saline solution. This fraction, almost completely precipitated by 0.55 to 0.67 saturation of ammonium sulfate, has an LD<sub>50</sub> of 0.6 to 2.0 micrograms on intravenous injection into 20 to 25 gram mice. Temperatures of 56° C. for 4 hours partially destroy it, and at 100° C. for 10 minutes the destruction is complete; it is stable over ranges of pH 5.0 to 8.0 and remains so for 4 months at 4° C. Formalin 0.1 per cent converts it into a

toxoid which retains antigenicity, while phenol in excess of 0.5 per cent, alcohol, mercuric borate and prolonged treatment with chloroform destroy it. The toxin of *P. pestis* is serologically distinguishable from the recognized atoxic antigens, which are precipitated by 0.33 saturation with ammonium sulfate. Both the toxin and toxoid are immunogenic in mice and rats. Purely antitoxic sera have been produced in rabbits. The Schwartzman phenomenon cannot be elicited with the toxin or any of the antigens.

Bacteriophages active against *P. pestis* have been described by a number of workers (Harvey, 1933). Lazarus and Gunnison (1947) demonstrated that a highly active strain of phage lyzed 12 out of 12 strains of *P. pestis*, but was also effective against 19 of 27 strains of *P. pseudotuberculosis*, 3 of 42 salmonella and 6 of 37 shigella cultures. After adaptation to *P. pseudotuberculosis*, the phage lyzed all 27 strains of that organism, but none of the *P. multocida* cultures examined. These and other recent findings attest to the uniformity of the antigenic structure of *P. pestis*.

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

In bubonic plague infections of man, *P. pestis* may be demonstrated in primary vesicles and with certainty in the gelatinous edematous fluid surrounding or within lymph nodes. Bacteremia at one stage or another of the disease is the rule, but its degree varies, in inverse relation to the interval before death, and with the time of observation. Even in mild cases of the bubonic disease, bacteremia is present; it may disappear as early as the second day or persist for 10 days in patients with severe infections. Kirchner (1934) made systematic blood cultures in 237 definite plague cases, obtaining positive blood cultures in 212. At autopsy *P. pestis* is regularly found in heart blood, lymph nodes or spleen, but especially

in bone marrow and secondary pneumonic processes. In primary pneumonic plague cases, the bacilli are present in sputa before they can be detected in the blood (Girard, 1929).

Experimental plague is produced in guinea pigs with ease by any route of infection; although usually susceptible to a few bacilli, these animals may show resistance during the winter months. They are invariably chosen for experimental purposes because of the characteristic lesions which they develop. However, it is not unlikely that their immunity differs from that of man. Many animal species are susceptible to spontaneous infections and respond readily to experimental infection by any route. Meyer (1947) gives a complete list of the more than 100 natural and suspected hosts of *P. pestis*. It should be emphasized that white rats and mice are not as susceptible as other *Muridae*, and that anatomic lesions produced in them are not as characteristic as those in guinea pigs. Otten (1941) claims the guinea pig to be the most susceptible rodent and the white laboratory rat the least susceptible. The wild rat (*Rattus rattus diardii*) and the white mouse he placed in intermediate positions. On the other hand, Sokhey and Maurice (1935) considered the mouse more susceptible than the guinea pig or the white rat! Monkeys are susceptible to plague, but individual variations in some species (*Macacus* and *Cynomolgus*) are quite common. The same conclusions apply to rabbits. The high susceptibility of the multimammate mouse (*Mastomys coucha*, Grasset, 1946) and the cotton rat (*Sigmodon hispidus*, Meyer, 1947) recommends them as test animals in plague work. Among the domestic animals dogs are usually unsusceptible; cats are moderately so. It has been claimed that sheep and camels may be infected. Birds are not susceptible.

The virulence of *P. pestis* when freshly isolated from human or rodent infections is, as a rule, of a high order; Barber (1912) found that 6 in 9 guinea pigs and 2 in 12

monkeys receiving one virulent bacillus died of plague. Organisms from cultures and occasionally chronic lesions in rodents may show a notable or complete absence of virulence. Strains losing part of their virulence will sometimes regain it upon passage through susceptible animals, although not all respond to this treatment. Many attempts have been made to reduce virulence of plague bacilli by growing them under unfavorable conditions (e.g., in alcohol broth), selecting at random colonies from virulent cultures refrigerated or subjected to incubation at 32° C. for some time (Otten, 1936), or by passage through murine animals (Burgess, 1930). Although losing virulence spontaneously, "natural" dissociants frequently retain the characteristics and immunogenic properties of parent strains, while "forced" dissociation mutilates the descendant by impairing its immunogenic powers and destroying the envelope. Russian investigators claim to have demonstrated an antigen similar to the Vi antigen of salmonella. Jawetz and Meyer (1943) have been unable to confirm the claim but admit the presence of some property or chemical group responsible for virulence in *P. pestis*, since some "natural" dissociants seem to be identical with virulent organisms in all respects except for a complete loss of virulence.

Standardized methods for measuring the virulence of plague cultures in mice and guinea pigs have been developed by Sokhey (1939) and Otten (1941). The virulence of a strain has no relation to the toxicity of the bacterial substance, but is rather an expression of its capacity to multiply in the tissues. Avirulent plague bacilli introduced in large numbers kill a high proportion of inoculated mice; in fact, certain avirulent strains are far more toxic than the virulent parent strains. In chick embryos, highly virulent plague strains proliferate freely, avirulent strains sparingly. The former in small numbers kill the embryo while the latter administered in sublethal doses per-



sist in embryonic organs until hatching time, and sometimes for 3 to 4 days after chicks hatch (Buddingh and Womack, 1941; Jawetz and Meyer, 1944b).

#### PATHOGENESIS

Essential for development of bubonic plague in man is the primary rat-flea-rat transmission cycle. The flea becomes infected from the blood of a sick rat, which in the terminal stages of bacteremia may contain  $10^7$  bacilli per cmm. of blood (Douglas and Wheeler, 1943) and then transmits the disease to another rat by its bite. Other rodents may replace the rat in the transmission cycle. The infection of man is an offshoot from the primary cycle, but bacilli are rarely present in human blood in sufficiently large numbers to infect fleas. Fleas infesting man, for example *Pulex irritans*, are not good transmitters of plague bacilli, and man thus constitutes a very weak link in the mammal-flea-mammal cycle of plague transmission. French epidemiologists disagree and continue to stress the importance of the human flea (Blanc and Baltazard, 1941). Thus, bubonic plague is never transmitted from man to man either directly or indirectly; it is not truly epidemic, though it may assume epidemic proportions. When plague bacilli become localized in the lungs and produce pneumonia, droplets from the respiratory tract are highly infectious. Under such circumstances, primary pneumonic plague may spread from man to man, and a true epidemic occurs. It has been claimed that plague bacilli inhaled with dust of infected flea feces induce primary plague pneumonia. In Manchuria infection has been traced to the eating of undercooked, plague-infected marmots. Primitive people who kill fleas by biting them between the teeth or who bite the heads off rodents have been infected via the alimentary tract.

*P. pestis* may enter the body via the blood, skin, conjunctiva or mucous mem-

branes of the respiratory and digestive tracts. In the infected rodent or man, both the lymph and blood streams may act as pathways for the bacillus. The pathogenesis of bubonic plague may be summarized as follows:

The plague bacilli ingested by a flea multiply in its midgut; many are passed in feces and may in rare instances get scratched into wounds. More frequently a massive infection develops in the proventriculus of the flea, blocking the pharynx and esophagus; when the flea attempts to take another blood meal a regurgitory effort ejects the bacilli (between 25,000 and 100,000) via the insect's proboscis into the skin or capillaries of the new mammalian host. This is probably the most important mode of transmission, though recent experiences incriminate a third. It has been established that while the flea is feeding on an infected host its mouth parts become contaminated, and direct transference of plague bacilli to a new host by this method has been proved.

The plague bacillus injected into the skin of the mammalian host, rodent or man, may be held up at the site; the local vesicle or pustule represents the first line of defense. It is seen in ambulant cases and in pestis minor, and reflects considerable individual immunity to the agent on the part of the host. Inflammation of the lymph channels leading from the local lesion to the lymph nodes is caused by toxins and not by the multiplying bacilli. If the *P. pestis* passes the skin barrier, it is held up at the first group of lymph nodes to which the lymphatic vessels pass; the nodes enlarge and are embedded in a gelatinous periglandular inflammatory edema. The whole process of infection may be arrested at this stage as the clinical type of pestis minor with only mild constitutional symptoms. If the bacilli pass the second line of defense, they reach the secondary lymph nodes draining the area of inflammation, and small numbers pass into the blood stream. Hence, there may occur invasions of the spleen, liver and other lymph nodes. An interplay between antibodies and fixed tissue leukocytes and

possibly other factors, may limit the bacteremia to showers. Nevertheless, generalized infection in many parts of the body creates grave constitutional symptoms. If the immunity mechanism is inherently inadequate or has been damaged by toxins, plague bacilli not only multiply intravascularly but are constantly washed into the circulation from the spleen and bone marrow, causing septicemic plague. In experimental plague, a highly susceptible animal which receives direct intravascular injection of plague bacilli through the bite of a flea may develop immediate septicemic plague with slight or only secondary involvement of the lymph nodes. Primary foci of bacillary multiplication in the spleen and bone marrow constantly flood the blood stream with organisms and toxins in the septic fulminating variety of plague. In the course of bacteremia, plague bacilli may localize secondarily in the skin and form pustules (carbuncular plague). Wherever plague bacilli multiply in enormous numbers, coagulation necroses with hemorrhagic inflammations seriously damage the lymph nodes and focal areas in the spleen and liver. These processes undergo slow resolution or suppuration, and may contain viable plague bacilli for many weeks. Lymph nodes may heal by softening and ulceration; in some instances there is gangrene (Jawetz and Meyer, 1947).

While Koulecha (1912) considered the tonsils or other parts of the upper respiratory tract to be portals of entry for the infection, which then travels to the lungs via the blood stream, most investigators believe that primary pneumonic plague is due to infection through some portion of the bronchi or bronchioles contiguous to lung tissues (Wu Lien-Teh, 1936). At least intranasal infections in mice and guinea pigs, experimentally produced, give rise to localized peribronchial and perivascular inflammation in tissues adjacent to bronchioles, and then to more diffuse inflammatory processes throughout the lungs. As Sprunts has indi-

cated, local toxin production reduces the resistance to infection; thus, in plague, confluent lobular and lobar areas of congestion and engorgement readily develop when bacilli reach the alveoli by extension from the peribronchial lymph patches (Meyer et al., 1947).

The characteristic picture at autopsy of human or animal plague is that of engorgement and hemorrhage associated with enlargement of the lymph nodes, and extravasation into the perinodular tissues. In severe cases the nodes may be in a state of serous or serosanguineous effusion, soft, and colored purple or plum-purple or completely infarcted hemorrhagically. In others there may be exudative edema of adipose tissue, fascia, muscles and nerve sheaths, while the hard nodes are studded with yellowish granules or colored with bright red extravasation. When the disease is protracted, necroses of varying degrees and yellowish-red pus are found in the parenchyma of the nodes; secondary buboes are often similarly affected. The spleen is enlarged and congested; on section the deep red or purple pulp has a granular appearance. The liver may be enlarged or engorged; its parenchyma is always soft, in a state of cloudy swelling or fatty degeneration. A large quantity of straw-colored fluid fills the pericardial sac. There are ecchymoses on the pericardium and endocardium; the myocardium is pale, soft, friable and degenerated. The blood is coagulated into soft clots or is in a semifluid state. Characteristic of the disease is the distention of veins and small blood vessels accompanied by large and small hemorrhages.

In all types of human plague the lungs are congested and edematous; in secondary plague pneumonia the areas of consolidation are reddish-gray and surrounded by a distinct ring of engorgement. Consolidated areas in animals are usually yellow. Regional lymph nodes are enlarged and soft. Punctate hemorrhages occur in the stomach and intestines. The swollen kidneys are pur-



tality from 5.5 to 75.0 per cent in different epidemics. More recent field trials by Sokhey (1936) with horse antiplague serum disclosed a case fatality rate of 28 per cent in serum-treated patients; and of 65 per cent in controls. Rabbit antiplague sera prepared in the United States with an avirulent strain of *P. pestis* showed high protective value for mice and white rats. They contain antitoxins and when concentrated as gamma-globulin solutions possess the highest protective value of any sera thus far tested (Meyer, 1947). All sera whether produced in rabbits, horses, sheep or bullocks, contain agglutinins, precipitins, complement-fixing antibodies, opsonins and mouse-protective antibodies. However, many are devoid of antitoxins, but despite this they protect rats against infection. For serologic studies, rabbits immunized with formalin-killed antigens yield antisera with titers over 1:1,280.

#### DIAGNOSIS

The diagnosis of sporadic cases is likely to be missed. Early diagnosis is of the greatest importance not only to the patient, whose recovery may depend on an early therapy but also to his family and to the community. In endemic regions it is imperative that the possibility of plague be considered at all times by physicians. A typical case of severe bubonic or septicemic plague presents a characteristic picture: sudden onset, high temperature, rapid pulse, white coating of the tongue, nervous symptoms varying from restlessness to great prostration and fatigue, bloated appearance and conjunctival suffusion, slurred speech and staggering gait, apathy and mental confusion. Eventually there is pain in the groin, armpit or neck, where the bubo appears. Intense pain directs the attention of the patient to the inflamed lymphatic node which may remain small, hard and tense, but more frequently is enlarged to the size of a walnut or goose egg and embedded in a boggy edematous swelling. In the septic variety

nervous and cerebral symptoms supervene with striking rapidity; although the temperature is rarely above 100° F., epistaxis, hematuria and involuntary evacuation appear in rapid succession. Pneumonic plague commences with rigor, malaise, severe headache, nausea, vomiting and general pain, temperature from 102° to 105° F., difficult and hurried breathing, cough and expectoration. The sputum, watery and frothy, becomes blood-tinged but is rarely viscid or rusty, as in acute pneumonia. An early etiologic diagnosis requires laboratory assistance.

**Laboratory Diagnosis.** By puncturing the bubo in its early stages with an 18-gauge needle mounted on a well-fitted 5 to 10 cc. syringe, a small amount of gelatinous, edematous fluid is aspirated. The operator should wear rubber gloves and mask. The skin over the bubo is painted with iodine, and the puncture wound disinfected with absolute alcohol. Care must be exercised in expelling the few drops of fluid on blood plates or blood agar slants and in making thin films on the several slides, since the spray may carry bacilli into the atmosphere. Polychromatic stain reveals the characteristic morphology of *P. pestis*. Small, delicate colonies develop in media incubated at 30° C. for 24 to 48 hours. Inexperienced persons should not attempt diagnosis from microscopic examinations alone, because contaminants such as *Escherichia* and *Salmonella* also give bipolar reactions. The culture may be quickly identified by means of specific bacteriophage, or preferably by an agglutination test with highly potent antiserum. For agglutination tests, the bacterial growth is emulsified in a 0.45 per cent saline solution containing 1 per cent formalin. The bacilli are killed in from 30 minutes to 2 hours at 37° C. The even suspensions, free from precipitates, are added to the serum. Flocculent agglutination usually appears within 2 hours at 37° C. Final identification is made by biochemical tests and guinea pig inoculations. Infection may

be accomplished by rubbing some of the culture intracutaneously, or by subcutaneously injecting a few drops of a heavy suspension. The animals will usually die within 3 to 8 days, with characteristic local and general lesions.

Blood cultures should be prepared by collecting at least 5 cc. of blood from the cubital vein and combining it with 2 cc. of sterile 5 per cent solution of sodium citrate. Enrichments are made in hormone-cystine broth or 0.25 cc. of the citrated blood is distributed on several blood agar slants or plates to reveal septicemia and its degree, if present. More than 10 colonies are indicative of established severe septicemia, while less may be interpreted as the temporary showers of mild septicemia. In the enrichment broth culture, chainlike aggregates of ovoid bacilli may confuse the microscopic picture. An agglutination test with the formalin-treated broth culture is useful.

Sputum should be examined both microscopically and culturally on blood or gentian violet (1:70,000) plates. There is usually no difficulty in recognizing the plague bacilli, which are present in great numbers.

Autopsy material should include heart blood, portions of bubo, spleen and bone marrow. Every state in the Union authorizes physicians or health officers to demand an autopsy if plague is suspected. Plague-infected material must be shipped in properly prepared containers with double screw tops.

#### TREATMENT \*

The value of antiplague sera prepared on horses has been repeatedly proved by members of the Haffkine Institute. Data illustrating its effectiveness have been recorded by Wagle (1941), and Sokhey and Wagle (1946), and Girard (1946). Mortality rate in treated cases was approximately 28 per cent, compared with 58 per cent in untreated controls; observers believed the degree of septicemia was the deciding factor

in the outcome of individual cases. To be effective, serum must be given early in amounts of from 60 to 160 cc., preferably intravenously. Attempts to develop potent sera which can be administered to man without causing alarming reactions have met with varying success. The rabbit antiplague globulin solution prepared in the United States is pyrogen-free and of high potency. Its use in man has not yet been reported. Principal objections to serum therapy are its expense and necessity for parenteral administration. Bacteriophage therapy, advocated by d'Herelle and used extensively in the French colonies, has yielded no clinical benefits (Sorel, 1947).

While all of the sulfonamides have been shown to have curative effects in isolated cases sulfadiazine is the drug of choice (Wagle et al., 1946). In pneumonic plague, dramatic recoveries have been reported following the use of sulfadiazine (Minter, 1945; Roux and Mercier, 1946). The drug must be given early in doses of 12 to 30 grams on the first day of disease, and in smaller doses for 12 days after the temperature is normal. Combined chemoserumtherapy, so effective in treating plague septicemia in mice (Meyer, 1947), deserves consideration in the light of equally encouraging observations on man made by Kamal et al. (1941). Streptomycin is the most effective therapeutic agent thus far discovered for the treatment of bubonic, septicemic and pneumonic experimental plague infections in mice and guinea pigs. It is recommended that human plague be treated, as soon as diagnosed, with daily doses of 2 grams of streptomycin in bubonic plague, and from 4 to 6 grams in the septicemic and pneumonic diseases; injections should be given at 4-hour to 6-hour intervals for the first 2 days. The dose may then be reduced, but in order to prevent clinical recurrence, treatment should be continued for at least 8 days on a 1-gram level, or substituted with adequate sulfadiazine therapy (Quan et al., 1947). Successful streptomycin treat-

\* Reviewed in Platzner, 1946.



ment of several human plague infections has been reported from Argentina.

#### EPIDEMIOLOGY

Plague is now endemic in certain parts of India; it is constantly present in Burma, Java, China, Madagascar and South, Central and East Africa. It occurs sporadically in Egypt, North Africa (Tunis), Irak, Iran, Siam and French Indo-China. Local rat epizootics, occasionally accompanied by a few human cases, have been noted in European seaports from time to time, the most recent ones being on the islands of Malta and Sardinia. In South America in the mountains of Ecuador, Bolivia, Peru and Argentina there are endemic foci. The United States has known rat and human plague since the beginning of the century: San Francisco (1900 and 1907), the Gulf States—Louisiana, Texas and Florida—(1914 to 1920) and Los Angeles (1924 to 1932) were in turn affected. After a 20-year hiatus, the rat disease erupted in Tacoma, Washington, in 1943 and 1944. Rural endemic foci are established on Maui and Hawaii. Human plague cases have been few since 1934 in California and the 13 western states extending as far east as Kansas and Texas, where pockets of enzootic and epizootic plague in ground squirrels, chipmunks, prairie dogs, wood rats and harvest mice have been recognized; in fact, since 1908 only 67 human cases with 43 deaths have been traced to wild rodent contacts in this area.

Since 1910 it has been recognized that there are two main epidemiologic groups of plague: (1) urban, domestic plague, and (2) sylvatic or rural plague. Either may develop from the bubonic to the pneumonic form, their epidemiology undergoing corresponding changes. Urban or domestic plague, transmitted almost entirely by rats, occurs in densely populated, insanitary areas, spreads along overland routes and crosses oceans in ship cargoes. Primarily

bubonic, it tenaciously remains fixed to human habitations, and spreads like an epidemic disease. Under certain environmental conditions, more frequently in cold countries, it becomes pneumonic, developing into epidemics through man-to-man transmission, as in Los Angeles in 1924. The sylvatic plague transmitted in rural areas and amongst hunters, workers in woods and children, through rodent bites or handling, and perhaps infrequently by insect bites, is primarily sporadic, bubonic plague (Meyer, 1942 and 1946). Cases in this epidemiologic group may also give rise to epidemics of pneumonic plague. Classic examples are the Manchurian epidemics of 1910 and 1911 and 1920 and 1921 which claimed 60,000 and 8,503 victims, respectively (Wu Lien-Teh, 1936).

The seasonal spread of plague is markedly influenced by temperature and humidity; a moderate temperature, 60° F., and a moderately high relative humidity indicated by a saturation deficiency of less than 10 millibars are the most favorable for checking it. The disease tends to occur during summer months in cooler climates and in the spring months in the hot, dry climates of the subtropics; in tropical countries where the temperature is fairly constant throughout the year, the incidence of plague follows the humidity curve. Many complex factors are involved in the periodic epidemic-like waves of plague, but fluctuations in its incidence in endemic areas are always associated with climatic variations. Persons of all ages and both sexes are equally susceptible to the urban bubonic type. General health, nutritional status and living conditions, including extent of overcrowding, are the determining factors in the mortality rates; there does not seem to be any racial immunity. Doctors and nurses who care for patients with pneumonic plague and adults attending funerals are liable to attack by the pneumonic form. In the rural, sylvatic plague regions men and

children who handle or play with wild rodents are more frequently attacked.

Man, the alternate host, contracts plague through the ecologic interaction of rats and fleas in his environment. Strains of *P. pestis* secured from man, rats, wild rodents and fleas from every corner of the earth are biologically identical and remarkably homogeneous with regard to virulence and infectiousness. Variations in the severity of epidemics, as for example in pneumonic plague, are not attributable to a specific pneumotropism.

The rodents involved in plague fall into two main groups: (a) The family *Muridae*, the rats, which live in close association with man and are responsible for the maintenance of endemicity and the epidemiclike outbreaks, and (b) the *Sciuridae*, *Cricetidae*, *Jaculidae*, *Cavidae* and *Leporidae*, generally known as wild rodents, which are the reservoirs of infection in sylvatic plague. Epizootics of plague among the large gray rats (*Rattus norvegicus*), the black domestic rats (*Rattus rattus alexandrinus*), and occasionally others, such as house rats (*Rattus griseiventer*) and field rats (*Rattus diardii* in Java and *Rattus hawaiiensis* in Hawaii), are influenced by the natural and acquired susceptibility of the individual rat, the density of the rat population, and the habits which by inclination or opportunity bring the species into close association with man.

Plague may be introduced into a rat population by "intramural" or contiguous spread from one section of a city to another, by spread at a distance through transportation of rats or rat fleas along lines of communication with merchandise, or by partial migration of rodents, as in Java. Unless there is a considerable population of *Rattus rattus*, plague is never transmitted to man to any serious extent. In San Francisco in 1907, 27 or 2.69 per cent of 1,002 live rats examined were infected; 56 human cases of plague with 25 deaths were reported during that time. By January, despite an incidence of 1.11 per cent infection among the rats of the city, only 2 cases of human plague were listed. Factors limiting the perpetuation of rat plague

are attributed to resistance against infection. High immunity rates, as for example over 50 per cent in San Francisco and over 90 per cent in Bombay, exert a fundamental influence, and may lead to the eventual disappearance of plague. Epizootic conditions cannot arise until a new generation of non-immune rats has grown up. It need hardly be emphasized that where houses are mainly constructed of soft material, and where garbage is carelessly disposed of, conditions are ideal for rat multiplication and explosive outbreaks of plague.

Endemic sylvatic plague foci maintained by burrowing, hibernating rodents such as tarbagans, *Citellus* varieties, spermophiles and mice are found in the brush, deserts and mountains of Manchuria, the Buriat Mongol Republic, Transcaucasia and South-eastern Russia; foci exist in South Africa among gerbilles and multimammate mice, in Argentina among *Microcavia graomys*, and in squirrels of the Peruvian-Ecuadorian frontier (Macchiavello, 1946). In the United States, sylvatic plague was discovered in 1908, and with the recognition of epizootics among squirrels in 15 western states has since 1934 assumed a new and intriguing position (Evans et al., 1943; Meyer, 1942). The collection from sick and healthy rodents of ectoparasites which were injected in emulsions into healthy guinea pigs demonstrated endemic and epizootic plague among 38 rodent species. Knowledge concerning the ecology of the species is quite fragmentary, and the conditions which in the past produced and now maintain these pockets of sylvatic disease are not clearly understood. The theory that they originated with the implantation of rat plague at the turn of the century has supporters (Tru-fant, 1944), while Meyer (1947) has maintained that sylvatic plague is far older than rat plague, and was probably enzootic on the American Continent before its arrival at West Coast ports.

The indispensability of the vector, the flea, in the rodent-flea cycle is well known.



The transmitting mechanism of the flea has been elucidated in recent years by Eskey and Haas (1940) and Douglas and Wheeler (1943). Only a comparatively small percentage of fleas feeding on an infected rodent with severe septicemia become infected, and a yet smaller percentage become infective, or capable of transmitting plague (Meyer, 1938). The numbers of infective fleas are conditioned by species, feeding habits, whether zoophilic (as are many wild rodent fleas) or anthropophilic, and the efficiency as a transmitter, which varies with species and is greatly influenced by climatic conditions. Very important is the longevity of fleas as preservers or "carry-over agents" of plague from one season to another (Joff, 1941; Prince, 1943, 1947). The climate and the size of the rat population control the density of the flea population; in urban situations density is measured by the flea index, which gives the average number of fleas on each trapped rat. Experience has shown that a "*cheopis*" index of at least 3 appears necessary for epizootic conditions. The most important and efficient vector of rat plague throughout the world is *Xenopsylla cheopis*.

*Xenopsylla astia*, a rat flea common in India, Ceylon and Mesopotamia, though capable of transmitting plague is relatively inefficient; when this flea predominates, the disease seldom reaches epidemic proportions and the carry-over to next season is limited. *Xenopsylla brasiliensis* and *X. nubicus*, which are found in tropical Africa, and *X. hawaiiensis* are known to transmit plague from rat to rat, but are relatively poor transmitters to man. *Ceratophyllus fasciatus*, the European rat flea, and *Leptopsylla musculi*, an inhabitant of the seaports of China and Japan, are relatively poor transmitters. Under special conditions the human flea (*Pulex irritans*) and the cat and dog flea (*Ctenocephalus*) have been incriminated as vectors (Jorge, 1928). Wild rodents have their various fleas, most of which are transmitters from rodent to rodent, and, under special circumstances, to man. In Russia, *Ceratophyllus tesquorum*, in South Africa, *Xenopsylla eridos* and *Dinopsyllus lypusus*, in Manchuria, *Oropsylla silantievi*, and in South America, *Rhopa-*

*lopsyllus cavicola* show the "blocking" phenomenon and imbibe human blood. Over 50 species of fleas infest the wild rodents of the western United States, most of them as susceptible to infection as domestic rat fleas (Eskey and Haas, 1940), but their vector efficiency has not been determined accurately (Burroughs, 1947). The common squirrel flea (*Diamanus montanus*) is an efficient vector, while *Hoplopsyllus anomalus* is a poor one. Rodent fleas bite man only when very hungry and, as a rule, attack their own hosts or another rodent rather than humans. It is therefore not unlikely that in sylvatic plague, flea transmission from rodent to man represents a weak link, accounting for few transmissions to humans. The microclimate in houses, ships and burrows greatly influences the species prevalence and density of the flea population (Stewart and Evans, 1941) which control the physiologic-pathologic processes leading fleas to block (George and Webster, 1934). Rodent lice and ticks have been found infected with plague bacilli but cannot compare with fleas as vectors.

Most investigators agree that primary pneumonic plague originates from a person ill with bubonic plague who has developed plague pneumonia of metastatic origin. This first pneumonic case leads to others as a result of complex factors. The patient may transmit the agent through droplets of sputum during coughing spells; *P. pestis* is known to be projected by cough for several feet from the face of the patient. An enormous mass of bacilli in the lung, overcrowding in badly ventilated dwellings, low temperature, undesirable social habits and customs, such as kissing of the sick, ignorance of precautionary measures against spread of infection—all these help bring plague to epidemic proportions.

#### CONTROL MEASURES

The standard methods of control aim at breaking the rodent-flea-rodent cycle. With the advent of the new insecticide, DDT (Kartman, 1946; Ludwig and Nicholson, 1947), and wartime experiences with chemoprophylaxis (Gordon and Knies, 1947),

procedures for the management of plague epidemics have undergone fundamental changes. In communities with histories of previous plague infection, preventive measures include prophylactic inoculation with killed antigens or with live avirulent cultures. One-dose immunizations are valueless, and whenever the risk is great, repeated inoculations must be made at monthly intervals. Persons entering suspected areas should not only be immunized, but should have their clothing and underwear dusted weekly with DDT. In infected areas, continual rodent and flea censuses must be carried out by live trapping of rats. The flea index and examination of rats for disease will furnish indications of any impending danger from plague. A program of this sort requires trained personnel: physicians, entomologists, mammologists, laboratory workers, teams experienced in the use of DDT and the disinfection of persons and premises.

When plague has been definitely diagnosed in a community, the patients and their immediate contacts receive first attention. Strict isolation in screened rooms and concurrent disinfection of all articles must be rigidly enforced. Physicians and nurses attending plague pneumonia or suspected pneumonic infections must wear hoods, masks with goggles, overalls and gloves. Contacts and suspected contacts of patients with pneumonic plague are first disinfected and segregated; their temperatures are then taken and chemoprophylaxis is instituted. From 3 to 8 grams of sulfadiazine should be administered each day for from 5 to 7 days. The population should be revaccinated even though contacts are protected by chemoprophylaxis. The usual quarantine procedures are applied to the area, with human and animal traffic rigorously controlled; in particular, the customary tendency of inhabitants of plague-infected regions to flee to neighboring communities must be prevented. Focal flea control must be instituted promptly. Areas

within a radius of about 200 yards around infected houses should be dusted with 10 per cent DDT; every person and domestic animal, as well as all clothing, bedding and furniture, must be dusted. Walls, ceilings and floors of houses are treated with DDT residual spray, rat runs and harborages with DDT insecticide powder (Ludwig and Nicholson, 1947). When the epidemiologic intelligence service through trapping of rats discovers new foci, disinfestation is extended, always proceeding inward from the outer zones of suspected infection. Personnel engaged in plague control should be protected by having their clothing impregnated with dimethylphthalate. Rat extermination with poisons such as "Antu" and 1080, fumigation with cyanogen gas, and other measures to reduce the rat population, including ratproofing, should be carried out after focal disinfestation. Vessels in contact with ports in plague areas should be protected against entry of rats, and cargo liable to flea infestation must be treated with insecticides. Epidemic control technics do not differ from those used in sporadic cases except for case-finding teams which search the area for unrecognized cases, and the enforcement of adequate isolation measures.

Whatever disagreement may exist regarding the magnitude of the menace of sylvatic plague, it is imperative to protect rural communities against exposure. The maintenance of so-called "rodent-free" belts around towns is imperative. Control of dense rodent and flea populations close to human settlements with the rodenticide 1080 (sodium fluoracetate) and DDT demands constant follow-up in order to be effective. To liquidate plague epizootics among susliks inhabiting over five million acres, the Russians undertook eradication measures on a gigantic scale. In 1939 they proudly announced that the *Citellus* population, which was 39 per 2.4 acres in 1934, had been reduced to 2.23. Such decimation of rodent reservoirs of plague liberates rural commu-



nities from the constant hazard of disease. In this connection, it is well to remember the words of Aubert Roche, "La civilization seule a détruit la peste en Europe, seule elle l'anéantira en Orient."

## PASTEURELLA PSEUDO-TUBERCULOSIS

### INTRODUCTION

The large, Gram-negative, elongated *P. pseudotuberculosis* organisms are pleomorphic, sometimes occurring in chains or in rodlike forms. They take bipolar stains, are motile when cultivated at from 18° to 26° C. but not, as a rule, at 37° C., and grow on media containing bile salts or in amino-acid solutions in the absence of accessory growth factors; they produce no gas in carbohydrates, no indole, and render litmus milk alkaline.

*P. pseudotuberculosis* causes spontaneous infections in rodents, pigeons, turkeys and canaries. In a small number of human infections it has been isolated from the blood stream during life, and from the characteristic necrotic lesions at autopsy. The mode of infection is not known. [Synonyms: *Streptobacillus pseudotuberculosis rodentium* (Preis, 1894), *Bacterium pseudotuberculosis rodentium* (Lehman and Neumann), *Bacillus der Pseudotuberculose* (Pfeiffer, 1889), *Bacillus parapestis* (Lerche, 1927), *Bacillus pseudotuberculosis rodentium* (Schütze), *Malleomyces pseudotuberculosis rodentium* (Pribram, 1933), *Yersinia rodentium* (van Loghem, 1944-1945).]

### HISTORY

The organism was first isolated by Malassez and Vignal (1883), who inoculated guinea pigs with material from a subcutaneous tubercular lesion on the forearm of a child dead of tuberculous meningitis. The animals developed nodules which, though histologically similar to tuberculosis, contain zooglycic masses of coccoid bacilli. Many investigators described under various names a bacillus causing a number of animal infections, which seemed identical with the "Bac. de Malassez et Vignal" (Dessy, 1925). Comparative study of the organisms

convinced Preisz (1894) that they were the same as those described by Pfeiffer under the name *B. pseudotuberculosis rodentium*. The agent has been encountered with increasing frequency in epidemic disease of barnyard fowls, cage birds, cats and monkeys. Because of its close relationship to *P. pestis*, first established by Galli-Valerio (1903), various strains of the agent have been the subject of detailed biochemical and serologic studies (Kaheki, 1915-1916, Schütze, 1928; Bhatnagar, 1940). Recent papers (Girard, 1942; Lazarus and Gunnison, 1947) stress the relationship of *P. pseudotuberculosis* to the enteric organisms, and that classification in the genus *Pasteurella* is anomalous.

### MORPHOLOGY

*P. pseudotuberculosis* varies in shape and size according to conditions of growth; it may be coccoid or ovoid, under 1  $\mu$  in length, or form definite rods 0.5  $\mu$  by 1.5 to 5.0  $\mu$  with rounded ends, either singly, in short chains or filaments. It is Gram negative but does not take bipolar stains as regularly as *P. pestis*. At room temperature (from 18° to 22° C.) and even in repeated transfers at this temperature, all typical strains show motility, never observed at 37° C. As a rule, from 1 to 3 flagella are demonstrable under favorable conditions; the large rods show peritrichous flagella (Weitzenberg, 1935). No spores or definite capsules are formed, though at 22° C. a viscous layer (or envelope) may be seen in India-ink preparations.

### CULTIVATION AND BIOCHEMICAL ACTIVITIES

*P. pseudotuberculosis* grows on ordinary media when freely supplied with oxygen. At 37° C. colonies are predominantly thin and dry, with irregular, rough edges, while at 22° C. they are moist, slimy and smooth. Shifts from R to S or intermediate types are probably due to the surface slime layer

which forms at different temperatures; they are not true dissociations. In broth, growth is diffuse at 22° C.; clumped masses and occasionally ring and pellicle formations are seen. The organism darkens meat and peptone by false pigmentation, but does not give color to synthetic media. It grows on gelatin without liquefaction but forms phosphatic crystals. Some strains develop in thin, yellowish-brown layers on potatoes. Slight but definite colonies appear on MacConkey's medium and desoxycholate agar.

Berkman (1942) noted that 5 strains of *P. pseudotuberculosis* grew readily in hydrolyzed gelatin or in his basal amino-acid medium, without accessory growth factors. Blood, glucose and adequate aeration yield heavy cultures.

Milk is not coagulated but alkalinizes quite rapidly. There is general agreement that the following carbohydrates and alcohols are fermented with production of gas: glucose, maltose, mannitol, galactose, arabinose, glycerol, isodulcitol, levulose, rhamnose, trehalose and xylose. No acid is formed in amygdalin, dulcitol, erythritol, inositol, inulin, lactose, raffinose or saccharose. Variations in fermentation of sorbitol, salicin and dextrin, both at 22° and 37° C., have been reported (Schütze, 1929; Topping et al., 1938). Cultures in peptone water containing 0.5 per cent glucose reach pH 4.6-4.8 at 37° C. in 7 days, but in the presence of 0.05 per cent glucose the final pH is 7.0 to 7.3. *P. pseudotuberculosis* forms no indole and gives a positive M.R. reaction. Nitrates and methylene blue are rapidly reduced. Small amounts of H<sub>2</sub>S are formed; the catalase test is positive.

#### ANTIGENIC STRUCTURE

There is general agreement that the H antigen is common to all strains but never forms at 37° C. (Bhatnagar, 1940). This antigen, associated with the flagella, agglutinates as loose woolly floccules, and is destroyed by boiling for 30 minutes, though

not by exposure to 56° C. Agglutination tests have also furnished convincing evidence that at least one of the somatic O antigens is common with *P. pestis* (Bhatnagar, 1940). *P. pseudotuberculosis* also possesses antigens which are present only in certain of its strains—the group-specific antigens. Reciprocal absorption reveals still another type-specific antigen, which reacts only with the strain against which the serum is produced. One of the somatic antigens is closely related to the O antigens of *Salmonella* (Schütze, 1928; Girard, 1943). The fact that phage lysis is not correlated with agglutination might be evidence that some strains of *P. pseudotuberculosis* have more than one antigen in common with *P. pestis* (Lazarus and Gunnison, 1947). Glycolipoid antigens have not been isolated (Girard, 1941). Claims that *P. pestis* spontaneously transmutates into *P. pseudotuberculosis* have not been confirmed.

Lysates or filtrates (Dessy, 1925; Meyer, 1928) of some *P. pseudotuberculosis* strains are lethal for mice, guinea pigs and rabbits; more sensitive to heat than similar preparations from plague bacilli, they resemble endotoxins.

According to Lazarus and Gunnison (1947) a strain of *P. pestis* phage lysed 19 out of 27 strains of *P. pseudotuberculosis* at 37° C., but susceptibility decreased on successive transfer at 22° C. due to the masking of somatic antigens. The *P. pestis* phage, readily adapted to *P. pseudotuberculosis*, remains active indefinitely on plague bacilli and may lyse certain strains of Shiga, Flexner, Sonne and Schmitz dysentery bacilli.

#### RESISTANCE

At low temperatures the resistance is great. Broth cultures are killed in 40 minutes at 60° C.; 5 per cent phenol and 60 per cent alcohol destroy the agent in 5 to 10 minutes. Cultures on blood media in sealed tubes remain viable for months, or as long as 30 years (Marlini, 1938). Streptomycin



inhibits rodent (rat and guinea pig) strains in concentration of 12.5 micrograms/cc. and 2 human cultures at 6.25 micrograms/cc.

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

Focal necroses in the liver, lymph nodes and spleen invariably yield cultures of *P. pseudotuberculosis* and in the early stages of infection bacilli are readily cultured from the blood stream. A great variety of animals serve as natural hosts. Pseudotuberculosis as a contagious disease is quite common in the birds of France and has recently been proved in canaries and in a blackbird in the United States. Outbreaks have been reported in chickens, chicks, pigeons and turkeys; sporadic infections occur in pheasants, swans, toucans, ducklings and in cage birds. It assumes epidemic proportions in guinea pigs and in rabbits. Sporadic infections have occasionally been encountered in horses, cows, goats, pigs, cats, hares, wild rats, baboons, chimpanzees and *Macacus monkeys* and man (Dujardin-Beaumetz et al., 1938). Many strains apparently have host preferences, with varying pathogenic affinities similar to those displayed by *P. multocida*. The experimental disease can be produced best by feeding guinea pigs, rabbits and mice; sparrows and canaries are quite susceptible, white rats usually refractory. *P. pseudotuberculosis* is claimed to be widespread in nature, having been recovered from soil, dust, water, fodder and milk (Schütze, 1929).

#### PATHOGENESIS

The mode of transmission is not definitely known, but it is believed that *P. pseudotuberculosis*, widely distributed in nature and disseminated through infectious excretions of affected birds or rodents, attacks susceptible animals through the digestive tract. As a rule, the abdominal viscera are primarily diseased. Injuries of the skin may

also serve as portals of entry. Young, inadequately fed animals are more susceptible than well-fed adults. Parenteral introduction of pure cultures is fatal to guinea pigs, which die in from 15 to 45 days, revealing at autopsy local abscesses, enlarged regional lymph nodes with caseous centers and white gray spots studding the spleen, liver, lungs and bone marrow. When bacilli are ingested, small necrotic nodules appear in the Peyer's patches of the ileum and cecum, and there is caseous necrosis of the mesenteric lymph nodes and omentum. In the course of epidemics, guinea pigs may exhibit 3 types of clinical manifestations: septicemia, fatal in 24 to 48 hours; classic pseudotuberculosis, in which there is emaciation, diarrhea and death in from 3 to 4 weeks; and the glandular form, with lymphadenopathy of the cervical and thoracic nodes, probably transmitted through bites. At any one of these stages the bacillus may be present in the blood stream. Severe septicemia is usually of short duration, and shows at autopsy acute splenic tumor, severe hemorrhagic enteritis and accumulation of clear fluid in serous cavities. Pathognomonic for *P. pseudotuberculosis* infections are the whitish nodules in the liver, spleen and occasionally in the lungs. They represent focal necroses composed of coagulated liver cells, granular oxyphil debris, and fragmenting polynuclear leukocytes which are frequently surrounded by foamy reticulum cells but rarely by epithelioid cells. Giant cells are always absent. The necrotic center may contain blood vessels plugged with bacterial emboli or remnants of vessels (Pallaske, 1932). Chronic lesions show extensive fibroblast and epithelioid proliferations which sometimes become granulomatous but never calcify. Bacilli are numerous in such lesions.

#### IMMUNITY

Guinea pigs that have recovered from the disease but possibly have a latent infection are immune to reinfection. Saisawa and

van Saceghem (1916) produced active immunity in guinea pigs with heated broth cultures. Failures reported by others must be attributed to the use of antigenically impotent cultures. Boquet (1937) successfully used avirulent cultures. Dessy (1925) was unable to demonstrate passive immunity in guinea pigs. The sera of recovered guinea pigs and of rabbits injected with silver-nitrate-killed antigens (Bhatnagar, 1940) agglutinate homologous and heterologous strains and contain complement-fixing antibodies (Bachman, 1921), opsonins and precipitins in varying concentrations. Infected and vaccinated animals may give a tuberculin type of delayed skin reaction on intracutaneous injection of dead bacilli.

Active cross-immunity for *P. pestis* attracted attention long before the existence of common somatic antigens had been discovered. Chloroform-killed, heat-killed or formalin-killed suspensions of *P. pseudotuberculosis* protect guinea pigs and rats against *P. pestis*. The soluble Fraction-1 antigen of the plague bacilli confers no immunity. Guinea pigs resistant to plague are still susceptible to pseudotuberculosis. Antiplague sera, as a rule, agglutinate a variety of strains of *P. pseudotuberculosis* to about the same titer; on the other hand, antipseudotuberculosis sera do not react with any of the *P. pestis* strains because they lack envelope antibodies (Bhatnagar, 1940). Cross-precipitation and cross-complement fixation experiments with *P. pestis* and *P. pseudotuberculosis* have given contradictory results. Antiplague sera do not confer passive immunity to guinea pigs against pseudotuberculosis.

#### DIAGNOSIS

The disease is diagnosed with certainty by bacteriologic examination only, since it cannot be distinguished either clinically or by anatomic changes from typhoid, paratyphoid, tularemia or tuberculosis (Rei-

mann, 1932). *P. pseudotuberculosis* is usually isolated on ordinary media without difficulty, and in pure cultures from the blood during life or from pathologic lesions at death. Differentiation from *P. pestis* and from *Salmonella*, which produce no gas, may be a matter of great difficulty. A number of media have been devised to simplify the identification, but none is absolutely diagnostic. Contradictory findings in cross-reactions between *P. pestis* and *P. pseudotuberculosis* may be due to antigenic variations as a result of temperature of incubation, presence or absence of an envelope, etc. Workers experienced with freshly isolated strains list the following characteristics, subject to modifications: (1) *P. pseudotuberculosis* grows more rapidly and more luxuriantly in transplants on artificial media than *P. pestis*; (2) motility of the pseudotuberculosis bacilli must be tested after repeated transplants at 22° C.—it is always absent at 37° C. after numerous transfers at this temperature; (3) *P. pseudotuberculosis* grows at 37° C. and rapidly alkalizes bromcresol purple milk; (4) *P. pestis* is virulent for the white laboratory rat, while *P. pseudotuberculosis* is not; (5) a specific antipseudotuberculosis serum does not react with *P. pestis*, but agglutinates heat-killed *P. pseudotuberculosis*. The serum of a patient may agglutinate bacilli isolated from his own blood in dilutions of 1:80 to 1:500 (Snyder and Vogel, 1943; Mason and Meyer, 1941); on the other hand, *P. pestis* is unaffected.

#### TREATMENT

Recovery of a patient from pseudotuberculosis following the use of sulfathiazole suggests that sulfonamides may have therapeutic value (Snyder and Vogel, 1943). Four strains of *P. pseudotuberculosis* isolated from different animal hosts proved resistant to as much as 1.5 units of penicillin per cc. (Quan et al., 1947).



## EPIDEMIOLOGY AND CONTROL

Nothing definite is known of the epidemiology of pseudotuberculosis in rodents or birds. Haas (1938) was unable to transmit the infection with rat fleas. Direct or indirect contact may introduce it into a flock of birds. Sporadic outbreaks of the disease during cold and wet weather amply attest the importance of predisposing factors. Protective vaccination has not proved successful. Hygienic conditions and prevention of exposure to infection are the usual prophylactic procedures adopted. When guinea pigs held in the laboratory are found infected, it is advisable to destroy the stock in the same room and to disinfect thoroughly the cages, tools and quarters.

## PSEUDOTUBERCULOSIS INFECTION IN MAN

Dujardin-Beaumetz et al. (1938) list 8 fatal infections in man, all beyond question pseudotuberculosis: 1 in Japan, 3 in Germany, 2 in Czechoslovakia, 2 in Austria and 1 in France. They also mention the case reported by Albrecht (1910) in which the resected appendix of a 15-year-old child, who had intimate contact with a cat, was proved by guinea-pig inoculation to contain *P. pseudotuberculosis*. To this list must be added 3 cases reported and one unreported in the United States (Topping et al., 1938; Moss and Battle, 1941; Snyder and Vogel, 1943; Mason and Meyer, 1943), and a mixed plague-pseudotuberculosis infection recorded by Macchiavello (1941) in Brazil. In the 14 cases vague prodromal malaise was followed by abrupt febrile onset with headache, chills, general pains and occasional catarrhal symptoms. The fever, of the irregular or "septic" type, at times reached 105° F. Anorexia, abdominal tenderness, constipation and variable degrees of leukocytosis were usual. Within a few days after onset the liver, and sometimes the spleen as well, became palpable and tender. Still other manifestations were sep-

ticemia, effusions into serous cavities, bronchitis, pulmonary engorgement and edema and parenchymatous changes in the liver, kidneys and pyocardium. Death was usually preceded by icterus, toxemia and stupor. Eleven cases terminated fatally between 10 to 18 days, one early in the third month. Diagnosis was established early by blood cultures and later by agglutination tests. At autopsy there were pathognomonic caseous necrotic foci, from 1 to 10 mm. in diameter, in the enlarged liver, spleen, mesenteric lymph nodes and occasionally in the pancreas. Hemochromatosis was frequently noted. Some patients exhibited enteric and colic ulcerations. With the exception of the case described by Macchiavello, all infections were of the abdominal type, a fact which supports the contention that they were acquired via the digestive tract. In two cases contact with cats or garden soil contaminated with their excreta (Albrecht, 1910; Paul and Weltman, 1934) are mentioned, while another patient had eaten rabbit (Moss and Battle, 1941).

TULAREMIA  
BACTERIUM TULARENSE

## INTRODUCTION

*Bacterium tularense* causes a specific infectious disease of wild mammals and ancillary hosts, in which it is maintained as a heterogenous infection through a variety of insects acting as reservoirs and vectors. Man may enter into the chain accidentally or occupationally by contaminating his hands, conjunctival sac or buccal cavity with the infected tissues or body fluids of certain animals, especially wild rabbits and hares, rodents, birds or insects, or by the bite of an infected blood-sucking fly or tick. Tularemia in man is an acute, febrile disease of moderate severity with a tendency to pneumonic complications; the clinical picture varies considerably according to the mode of entry of the agent. [Synonyms: *Pasteurella tularenensis* (Bergey et al.), *Brucella tularenensis* (Topley and Wilson, 1931) and *Bacillus tularenensis* (Kelser, 1931).]

## HISTORY

Tularemia is not a new disease. The adaptation of the parasite to rodent ectoparasites suggests that it is an infection of considerable antiquity, which has been endemic on the American Continent for a long time. G. W. McCoy in the course of his studies on plague, in 1910, discovered among ground squirrels (*Citellus beecheyi*) a disease characterized by pathologic lesions similar to those of plague. This disease was encountered in rodents shot or found dead in Tulare County, California, and McCoy and Chapin (1911) named the causative organism *Bacterium tularense*. Wherry and Lamb (1914) recognized bacteriologically the first infection in man and established the hare as an important source of the disease. Francis in 1919 and 1920 investigated rabbit fever in Utah and discovered that the blood of an infected rancher, who had been bitten on the neck by a deer fly, produced the same plaguelike disease in guinea pigs. In rapid succession he reported the isolation of *Bacterium tularense* from jack rabbits, the transmission of the disease by the bites of the deer fly (*Chrysops discalis*) and rabbit louse (*Haemodipsus ventricosus*), the cultivation of the organism on a new medium, and the means for serologic diagnosis. The importance of ticks as reservoirs and vectors of *Bacterium tularense* has been elucidated by Parker and Spencer (1924, 1926 and 1929) and by Green (1929). Most of what is known about tularemia was established by the extensive field, laboratory and clinical investigations of Francis and his colleagues in the U. S. Public Health Service. The cytotropism of the bacterium was first recognized by Francis (1927) and its significance established by Buddingh and Womack (1941). Foshay and his colleagues (1934, 1940) developed an antiserum and a vaccine.

## MORPHOLOGY

*B. tularense* is extremely polymorphous (Fig. 23). Small coccoid forms occur in in-

fect heart blood, while cultures exhibit coccoid and cylindrical or flattened bacillary forms, globules and globi, filaments and minute reproductive elements. Coccoid forms range from 0.5 to 3.5  $\mu$  in diameter; bacillary or ribbon-shaped forms also vary in size. Budding gives rise to minute reproductive units, 300 to 350  $m\mu$  in diameter.

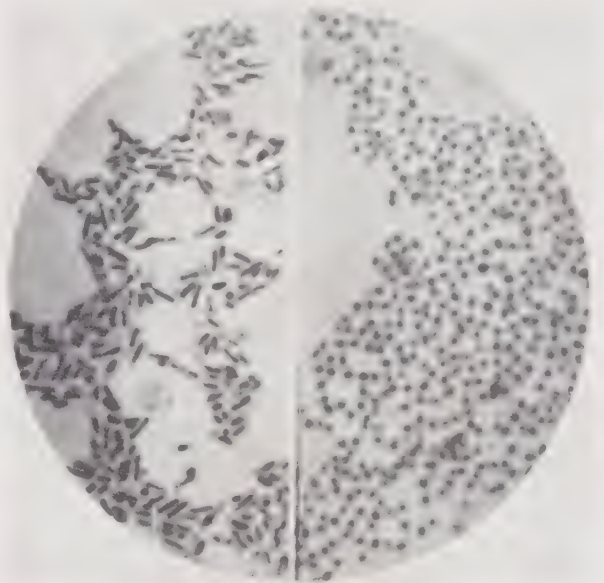


FIG. 23. *Bacterium tularense*. Note change from coccoid form to bacillary form in 24 hours following a single transfer to fresh culture medium. (Photomicrograph obtained from E. Francis, Army Institute of Pathology. It has already appeared in Stitt, E. R., and Strong, R. P., 1942, Diagnosis, Prevention and Treatment of Tropical Diseases, ed. 6. Philadelphia, Blakiston, p. 715.)

which are filterable. *B. tularense* possesses neither capsules nor flagella and is non-motile (Hesselbrock and Foshay, 1945). Dilute carbolfuchsin or gentian violet, or preferably a polychromatic eosin-methylene blue preparation, will stain the organism in smears and sections.

## CULTIVATION AND BIOCHEMICAL ACTIVITIES

On semisolid media such as gelatinized yolk of hens' eggs (McCoy and Chapin) or on glucose-rabbit-blood-cystine agar (Francis), *Bacterium tularense* forms minute,



transparent, droplike colonies that are mucoid and easily emulsified. Media inoculated with infective tissue may show discrete growth in from 2 to 7 days, but in subcultures confluent growth appears in 24 to 48 hours. Routine blood cultures may be obtained in Rhamy's hemoglobin-cystine agar or thioglycolate heart-infusion agar. Luxuriant growth of fully virulent cells can be obtained in consecutive transfers and with a variety of media provided large inocula are used (Berkman, 1942; Tamura and Gibby, 1943; Larson, 1945b; Snyder et al., 1946). Decreased oxygen tension or large inocula are required to initiate growth. It develops slowly in hydrolyzed gelatin media containing thiamine and requires also thermostabile, dialyzable accessory growth factors derived from liver cake extract; blood cells or biotin concentrates are necessary for growth in amino-acid solutions. Cystine or cysteine are indispensable. A salt concentration of 2 per cent is not inhibitory. The optimum temperature for growth is 37° C. Growth occurs readily in yolk sacs and on membranes of embryonated hens' eggs (Downs et al., 1947).

Francis (1942) found that glucose, maltose and mannose are fermented without gas production, while fermentation of glycerol, levulose and dextrin is irregular.

#### ANTIGENIC STRUCTURE

Several strains of *B. tularensis* examined by various workers appear to be antigenically homogenous. Francis and Evans (1926) found that a serum prepared against *B. tularensis* agglutinated *Br. melitensis* and *Br. abortus* in titers one-quarter to one-sixth of the original. Both types of *Brucella* absorbed homologous agglutinins from tularensis sera. *B. tularensis* was agglutinated in low titer by antimelitensis and antiabortus sera but was unable to absorb the homologous agglutinins from them. There are no cross-immunity relationships with *P. pestis* or *P. pseudotuberculosis*. Avirulent strains

of *B. tularensis* occur through natural dissociation in the course of regular transfers in glucose-cystine agar (Francis, 1929; Foshay, 1940). Long-continued residence of the organism in birds also tends to lower its virulence (Green, 1943).

No soluble toxin has been demonstrated by workers in the United States (Tamura and Gibby, 1942); however, Khatenever (1943) in Russia claims that cultures on semisolid colloidal media yield sterile filtrates which produce toxic reactions in white mice. The bacterium has also been reported to contain an endotoxin (Öz, 1940). A heat-stabile fraction which gives skin reaction is present in cultures grown in yolk sacs (Larson, 1945a).

#### RESISTANCE

The agent has great surviving power, having survived in cultures for 22 years at 10° C. without transfer (Francis, 1943), in carcasses for 133 days, in hides for 40 days, in water up to 3 months (Khatenever, 1943), in pure glycerin at -14° C. for 2 years, and at -70° C. for months. Dried in vacuo and held at room temperature, it is viable for 4 years (Miller, 1946). Heating at from 55° to 60° C. for 10 minutes or cooking of infected tissues kills the organism. The usual disinfectants inactivate it: tricresol 1 per cent, in 2 minutes, 0.1 per cent formalin in 24 hours and 0.4 to 1.0 ppm. of chlorine in 15 minutes (Foote et al., 1943).

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

In man, *B. tularensis* may be recovered during the first week of disease by inoculation of guinea pigs, or by direct culturing into suitable media. Pus taken from suppurating lymph nodes early in the disease contains viable bacteria, but the late adenopathies are usually sterile. At autopsy in man and in wild and laboratory animals the

acute necrotic lesions in the liver, spleen, lungs and bone marrow readily yield positive cultures. Inoculation of guinea pigs with tissues from field mice occasionally establishes the presence of the infective agent when there is no anatomically visible latent disease (Burroughs et al., 1945). That *B. tularensis* is intracellular in the liver cells of infected mice, as well as in epithelial cells of the rectal sac and malpighian tubes of ticks and lice, reflects the close adaptation of the organism to its hosts. It explains in part the persistence of the bacilli in organs of mammals, and transovarian transmission in ticks. The naturally infected vertebrate hosts belong to the orders *Rodentia*, *Insectivora*, *Carnivora*, *Ungulata* and the class *Aves*.

Burroughs et al. (1945) list 48 different species including the principal hosts, the cottontails (*Sylvilagus floridanus*), jack rabbits and snowshoe rabbits (*Lepus spp.*). In North America the cottontail rabbit (*Sylvilagus spp.*) is the direct source of over 70 per cent of all human cases of tularemia (Jellison and Parker, 1945). Other animal sources of human infection are gray squirrels, fox squirrels, opossums, woodchucks, muskrats, skunks, coyotes, foxes, cats, sheep, deer, water rats (*Arvicola amphibius* in Russia), voles and bull snakes. Wild rats, meadow mice, several species of squirrels, chipmunks, lemmings (Olin, 1942) and domestic calves have been found naturally infected, but so far have not caused human disease. Game birds, quail, prairie chickens, pheasants and domestic chickens are among the avian sources of infection. Two species of grouse, the sage hen and the horned owl have been found naturally infected, but no human infections have been traced to them. *B. tularensis* is highly infectious for white laboratory mice and rats, guinea pigs, rabbits, hamsters, monkeys and even cold-blooded animals. Certain species of ticks are important reservoirs of the disease. It spontaneously infects *Dermacentor andersoni*, *D. occidentalis*, *D. variabilis*, *Ixodes ricinus*, *Californicus*, and survival in at least 54 arthropods has been reported (Steinhaus, 1946).

The ticks directly related to human disease are the Rocky Mountain and Western wood ticks, the common Eastern dog tick,

the Lone Star tick (*Amblyomma americanum* in Tennessee), the deer fly (*Chrysops discalis*) and the mosquito (*Aedes cineris* in Sweden). *B. tularensis* is known to survive under many different environmental conditions, in still water as well as in streams (Karpoff and Antonoff, 1936; Jellison et al., 1942), moist soil, hides, infected carcasses and food products. One stream contamination in Montana persisted for 33 days.

#### PATHOGENESIS

*B. tularensis* has no specific portal of entry. According to Francis (1937) there are at least 20 known methods by which man can be infected. The most important are: contact with infected vertebrates and discharges of arthropods; accidental transmission through bites of mammals, particularly carnivores, and arthropods; ingestion of partially cooked infected meat of vertebrates or of infected water; inhalation of aerogenically dispersed material from cultures, infected laboratory animals, and fecal droplets of ticks.

When the bacteria penetrate the skin or mucosa, there develops in about 10 per cent of the human infections a papulelike primary lesion which soon ulcerates. The lesion appears on the hands, arms, face, or in the conjunctiva, but has been observed in the oral and pharyngeal cavities and in the nares. There is usually an initial bacteremia for not longer than a week. However, in highly susceptible experimental animals and in some humans the disease takes the form of a septicemia from its onset, or develops into a severe one resulting in early death from the fourth to the eleventh or twelfth day. From the site of inoculation, the bacteria spread along the superficial and deep lymphatics, leading to dermal lymphangitic nodules, lymphadenitis and bubo formation in more than 90 per cent of the human infections. In the absence of discoverable primary lesions, lymphangitic invasion is rare. The transitory bacteremia gives rise to dif-



fusely scattered foci of necrosis in the spleen, liver, lungs, lymph nodes, bone marrow and possibly other tissues and organs. With the appearance of antibodies bacteremia disappears, but new lesions may develop by lymphatic extension. A second, invariably fatal invasion may occur, which usually disperses bacteria through both the systemic and pulmonary circulatory systems, resulting in the formation of miliary and submiliary foci of necrosis in nearly every organ (Foshay, 1937). The typical primary ocular lesion is an ulcerated papule located on the lower lid, which is followed by general infection of the conjunctival sac characterized by congestion of the vesicle, lacrimation, damage to the eye and involvement of the lymphatics. Ingestion of *B. tularensis* causes violent local disease processes; necrotizing pharyngitis, abscesses in the roof of the mouth, enlargement of the submaxillary and cervical lymph nodes, and ulcers, hemorrhages and minute necroses in the gastro-intestinal tract. There is always fever, averaging 31 days in duration, which usually shows an initial rise, a remission and a secondary rise. The sedimentation rate is elevated, but there are no characteristic blood findings; the leukocyte counts vary from 5,000 to 20,000 with relative or absolute polynucleosis. Primary pulmonary tularemia is not widespread, but, according to recent reports, the incidence is on the increase. Tularemic pneumonic consolidations resemble tuberculosis, being characterized by confluent or lobular bronchopneumonic necrotizing infiltrations of the parenchyma, which may be followed by cavitation, gangrene and pulmonic abscesses.

The gross lesions of tularemia vary somewhat in different animals, but they resemble very closely bubonic plague in the guinea pig. The site of inoculation is necrotic, the contiguous lymph nodes hemorrhagic and caseous; the liver is covered with yellow-gray necrotic foci and the spleen studded with thick miliary necrotic granules. Chronic tularemia in guinea pigs and squirrels is

marked by enlargement of the lymph nodes and the presence of necrotic foci resembling pseudotuberculosis. Spontaneous tularemia in rabbits gives rise to necrotic foci in the spleen and liver, the lymph nodes are free of lesions. Findings are similar on mice; in this species the spleen alone shows macroscopically visible nodules. The relatively resistant white rat may show an enlarged spleen and few necroses. At human autopsy, tularemia lesions are generalized, being found in the skin, conjunctiva, lymphatic structures, and the tissues of the reticulo-endothelial system; there are necroses in the spleen and liver. The lungs may be normal, show discrete nodules, extensive necrosis or large confluent areas of bronchopneumonia. The heart seldom shows gross abnormalities; the myocardium is pale, soft and flabby. Fibrinous pericarditis, thrombophlebitis, fibrinous exudates in the peritoneum, meningitis and encephalitis have been noted.

The histogenesis of the typical lesions is characterized by a rapid accumulation of mononuclear wandering cells, principally macrophages; polymorphonuclear cells play little part in the reactive processes. The fundamental cellular reaction depends upon a complex biologic and physical relation between the bacteria and the host cells, leading to necrosis, in which there is an interplay of reticulo-endothelial-monocyte-epithelioid types of cells. Vascular changes, though conspicuous in the usual advanced lesions, are not responsible for the necroses, nor is anoxemia (Francis and Callender, 1927; Lillie et al., 1936). Of pathologic significance are the capacity of *B. tularensis* to multiply within hepatic and endothelial cells of guinea pigs and its selective affinity for thin ectodermal cells (Buddingh and Womack, 1941). These facts certainly account for many features characteristic of acute and chronic tularemic infections. Human autopsy material must be re-examined in the light of the newer concept that *B. tularensis* is a facultative intracellular para-

site, which may persist for years in the organs in a latent stage (Foshay and Meyer, 1936).

#### IMMUNITY

An attack of tularemia confers a pronounced immunity, and when a secondary infection occurs it is usually not severe. Thus, butchers having suffered one attack are not known to experience others. Francis observed a laboratory worker who developed on a crack in his finger a small papule containing *B. tularensis* 29 months after an initial attack. The patient had neither fever nor constitutional disturbances, and only slight enlargement of epitrochlear and axillary lymph nodes.

Rats, rabbits and other laboratory animals which occasionally recover from experimental infections as a rule are resistant to many fatal doses. The problem of active immunization is intimately connected with the susceptibility of most rodents and rabbits to infection. While Francis and others never succeeded in protecting guinea pigs and mice with inoculations of heat-killed or chemically killed antigens, cultures, filtrates or avirulent cultures, Downs (1932) et al. found that domestic rabbits immunized with formalin-killed cultures of *B. tularensis* developed a slight resistance to subsequent infections. Since the susceptibility of the white rat is low in comparison to that of rabbits, Larson (1945a) tested the response of this animal to different types of antigens prepared from infected yolk sacs and to avirulent strains and found that the aqueous phase of ether-extracted yolk vaccine produces active immunity. Attempts to immunize mice (Downs et al., 1947) and monkeys (Coriell et al., 1947) with different types of antigens resulted in protection against only small, infective doses. Foshay et al. (1932) vaccinated 2,145 persons with oxidized virulent strains of *B. tularensis* grown on artificial media and succeeded in producing agglutinins without provoking local and general reactions. Although this

vaccine failed to protect rabbits against a strain of maximal virulence, Foshay et al. (1942) concluded that it confers a "useful degree of protection," evidenced both by a decreased incidence of infection and by amelioration of the clinical disease in those who were attacked.

Antisera prepared in rabbits, sheep, goats and horses protect mice against 10,000 M.L.D. when administered simultaneously with infective organisms, but are of no therapeutic value when given 24 hours later. Sera produced from goats and horses by prolonged subcutaneous inoculation of formaldehyde-killed suspensions of virulent strains have been extensively used in the treatment of human tularemia; Foshay (1940) gives statistical and clinical evidence that they modify favorably the course of the disease. Infection or active immunization provokes the appearance of agglutinins and complement-fixing antibodies. The aqueous phase of 10 per cent infected yolk suspensions inactivated with formalin or phenol serves as an excellent antigen in complement-fixation tests.

Infected animals and man give delayed, tuberculinlike hypersensitive reactions on intradermal injections of oxidized bacillary suspensions.

#### DIAGNOSIS

Certain circumstances should lead one to suspect that a febrile illness is tularemia, e.g., when the patient has killed, skinned and cleaned rabbits, or has been bitten by deer flies, other arthropods, or by a mammal in an endemic area. In many cases, however, the mode of infection is completely obscure. Other tularemia signs are a febrile, influenzalike attack showing an initial sharp rise, a temporary remission, and a further febrile bout of about a fortnight's duration, followed shortly by a local lesion, possibly conjunctivitis, and tender, enlarged lymph nodes. The pneumonic type is particularly difficult to diagnose clinically, and appears like an atypical pneu-



monia which does not respond to chemotherapy.

**Intradermal or Percutaneous Allergic Skin Tests.** The intracutaneous injection of 0.05 cc. of a specially prepared detoxified suspension of killed *B. tularensis* is a valuable diagnostic aid. A tuberculin type reaction appears within 48 hours. It is almost always positive during the first week of disease when agglutinins are still absent. Skin test reactions persist for many years after recovery from the disease, and cannot be used as an index of recovery (Foshay, 1932). Intradermal injection of a minute quantity of antitularensis serum causes an immediate erythematous-edematous reaction, which has been shown to be bacterial specific. Since it is in a way related to serum protein sensitivity, it is useful as a quick confirmatory aid (Foshay, 1936).

**Agglutination and Complement-Fixation Test.** Specific antibodies never appear in the blood during the first 10 to 12 days of the disease. They almost always appear during the second week, rising abruptly in the third week, reaching maximum titers on the fourth and fifth weeks. A rise in titer is almost invariably conclusive proof of tularemic infection. Positive agglutination may persist for the remainder of life of the patient. Hence, a positive agglutination test does not indicate disease in the absence of symptoms and signs of tularemia. As a rule, a tularemia serum agglutinates *B. tularensis* to a much higher degree than *Br. abortus* or *Br. melitensis*.

**Cultures** should be made on dextrose-cystine agar or thioglycolate blood agar; guinea pigs, mice or chick embryos should be inoculated with specimens of blood, pleural effusions, exudates from primary lesions or sputa from living patients, or with heart blood taken at autopsy. When agglutinins are absent, *B. tularensis* may be isolated from the sputa of persons suffering from tularemia who manifest no frank clinical signs of pulmonary involvement (Larson, 1945c).

## TREATMENT

Until the advent of streptomycin, serum therapy was the only form of treatment that had been studied on a large scale. Today, streptomycin approximates the ideal therapeutic agent for tularemia. Multiplication of the bacterium is prevented by 0.4 microgram/cc. in vitro when exposed for 2 hours. Single doses of 10 micrograms/Gm. body weight protected 92 per cent of mice against simultaneous challenge with 15 to 20 M.L.D. of a strain of maximal virulence. When therapy was delayed for 72 hours after challenge, 80 per cent survival was obtained with 10 micrograms/Gm. every 3 hours for 10 days (Chapman et al., 1946). Streptomycin has a usually prompt and often dramatic effect on the course of the human disease, with few untoward reactions. The amazing effectiveness of 0.5 Gm. per day for 6 days even in tularemic pneumonia justifies the expectation that death can be prevented if treatment begins early enough. The formulation of an optimal dose depends on further clinical experience (Foshay, 1947).

## EPIDEMIOLOGY

Tularemia has been reported from all parts of the United States; some areas are much more heavily infected than others. In five New England states only 20 cases have been reported since the discovery of the disease. Vermont is the only state so far entirely free of it. Francis (1942) records by states 14,002 cases of tularemia; of these, 9,171, or 65.5 per cent, occurred in states where cottontail rabbits are the principal sources of infection (Jellison and Parker, 1945). Tularemia has been recognized in many other parts of the world.

Tularemia is an essentially sporadic disease. It may appear in epidemic form when a number of persons take an infected meal, as in the cases reported by Amoss and Sprunt (1936) with 12 deaths in 20 victims;

when contaminated drinking water is consumed (Karpoff and Antonoff, 1936); or as it did in a boys' camp when 30 of 170 boy residents were infected by deer-fly bites (Hillman and Morgan, 1937). No other disease has such a variety of modes of transmission, but it is doubtful if the infection is ever transmitted from man to man directly or indirectly.

Natural infection occurs in every month of the year but is most prevalent in the summertime in the Western States. There, ticks are particularly dangerous from March to August, deer flies from June to September. Jack rabbits are hunted particularly from April to October and are a threat at that time. In the Eastern States, cottontail rabbits are hunted during the months of November to January, and are the source of seasonal infections.

Men and women of all races and all ages are susceptible. Hunters, farmers, campers, housewives, butchers and laboratory workers are among those most commonly infected. The highest annual incidence on record is that of 261 cases in 1939. The mortality rate in 15,525 cases reported in the United States up to 1942 was 6.9 per cent. In Russia the case fatality rate is 1 per cent.

The key factors in the transmission of tularemia are ticks and vertebrates belonging to the *Rodentia* (*Leporidae*, *Muridae* and *Sciuridae*). For its propagation in nature *B. tularense* depends on the wood ticks (*Dermacentor andersoni*) and the dog ticks (*Dermacentor variabilis* and *D. occidentalis*, and *Haemaphysalis leporis palustris*), which feed on rabbits and other rodents. The chain of infection is efficiently maintained: ticks in all stages of development, from larvae to adults, are transmitters;

adult females pass it to succeeding generations by transovarian transmission. The multiple factors affecting the bionomics and ecology of the insect and rodent reservoirs vary from region to region and from country to country, creating a complex, ecologic picture which thus far has been sketched only in its broadest outlines.

#### CONTROL MEASURES

Endemic tularemia of rodents cannot be eradicated. An increased incidence of tularemia always coincides with an increase in the rodent population, whose numbers decline when the regulator—*B. tularense*—has been active. Under certain circumstances, poisoning campaigns are indicated, e.g., as against water rats and mice in Russia. Some reduction in the incidence of tularemia would be accomplished by supervising interstate shipments of wild hares, and their sale for food in markets and restaurants. Until the prophylactic value of vaccines has been more conclusively proved, it is imperative that sportsmen, butchers and those who live in regions where the infection prevails should be educated to the dangers of this disease. To render meat of rabbits harmless, thorough cooking is necessary. Rubber gloves should be worn while dressing rabbits. Drinking water from streams in endemic regions should be avoided. Non-immune laboratory workers should be protected by face masks and rubber gloves. Isolation is not necessary, but discharges from suppurating local lesions must be disinfectant.

Early diagnosis and treatment with streptomycin will in time reduce the case fatality rate.



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## 20

# The Brucella

### INTRODUCTION

The brucella are Gram-negative, rod-shaped, nonsporulating and nonmotile aerobic bacteria which may be coccoid in shape. They often require increased concentrations of carbon dioxide upon primary isolation from infected hosts. They do not produce visible acid or gas on fermentation. They live a strictly parasitic life and characteristically invade mesenchyme cells of various organs of man and animals.

Three species are found in manifest and latent infections: *Brucella melitensis* (synonyms: *Bacterium melitense* Bruce, *Micrococcus melitensis*, *Alcaligenes melitensis*, caprine Brucella), *Brucella abortus* (synonyms: *Br. melitensis* var. *abortus*, *Alcaligenes abortus*, abortion or Bang's bacillus, bovine Brucella) and *Brucella suis* (synonyms: *Br. melitensis* var. *suis*, porcine abortus strain). The disease in man is commonly known as undulant fever (synonyms: Malta fever, melitococcie, Mediterranean fever, rock fever of Gibraltar, Texas fever, Rio Grande fever, Neapolitan disease, Bang's disease, brucellosis).

### HISTORY

Precise information concerning brucellosis began with the description of the clinical signs by Marston in 1859 and the discovery of the etiologic agent in the spleen at necropsy by Bruce in 1887. The isolation of the organism, then called *Micrococcus melitensis*, from the milk and urine of infected goats on Malta (Zammitt, 1906) and the demonstration of natural infection in sheep

were paralleled by Bang's studies on infectious bovine abortion which led to the isolation of the second species, then called *Bac. abortus* (Bang, 1897). The third species, *Br. suis*, was isolated from swine by Traum in 1914. For many years the 3 species were regarded as unrelated until the relationships between the *abortus* and *melitensis* organisms were revealed by Evans (1918). The 3 species were then placed in the newly created genus, *Brucella*.

### MORPHOLOGY

Although the definition of the genus *Brucella* refers primarily to rod-shaped organisms, coccobacilli, coccoid forms or rod-shaped forms may predominate in either *abortus* or *melitensis* cultures. In an analysis of over 300 strains of brucella, it was shown that only 6 per cent of the *melitensis* cultures and 46 per cent of the *abortus* cultures were predominantly rod shaped (Wilson, 1933). If primary cultivation from animal exudates or tissues is performed in rich media or in media with a surface tension exceeding 55 dynes, the coccoid form is abundant in *melitensis* culture, whereas, under the same conditions, the bacillary form of *Br. abortus* and *Br. suis* predominates. On tryptose agar the organisms appear to vary in length from 0.5 to 2  $\mu$  and in width from 0.4 to 0.8  $\mu$ .

Capsules have been demonstrated on the



smooth and intermediate variant forms. It has been recently reported that the capsule plays a definite role in determining antibody specificity, although this remains to be confirmed.

The organisms stain easily, are Gram negative and non-acid fast. They may show granule formation under certain conditions, such as vigorous aeration of the culture.

### NUTRITION AND METABOLISM

The organisms grow well on ordinary laboratory media when once adapted to a lifeless environment. Their primary isolation is, however, not as easy to accomplish. Various media based upon liver infusion and "tryptose" peptone supplemented by dextrose or glycerine, yield luxuriant growth. For the growth and preservation of cultures, a medium containing 2 per cent glycerine, 1 per cent dextrose, 2 per cent tryptose, 0.3 per cent meat extract, 0.5 per cent sodium chloride, adjusted to pH 6.8, is very satisfactory. Nutritional requirements include cystine, histidine, tyrosine, phenylalanine, and tryptophane, thiamine and niacin; calcium pantothenate and biotin also stimulate growth as well as magnesium, manganese and iron. *Br. abortus* requires 5 to 10 per cent carbon dioxide tension for primary isolation, whereas the other two species can grow at atmospheric carbon dioxide tension. After adaptation to laboratory media, however, *Br. abortus* strains usually lose their specific carbon dioxide requirement.

Growth of very small inocula is readily obtained in the 9- to 11-day-old embryonated hen's egg.

Carbohydrates are utilized by all species, but production of visible acid and gas does not occur. All species reduce nitrates and produce catalase. *Br. melitensis* usually does not produce  $H_2S$ ; *Br. abortus* produces it for about 2 days; and *Br. suis* produces it for 4 days and longer. However, reliance cannot be placed on any one single meta-

bolic test to classify strains, since gradations in properties occur among the species.

### RESISTANCE

*Brucella* are destroyed quickly by hypochlorites, benzalkonium chloride, merthiolate, phenol and formaldehyde in concentrations effective against other Gram-negative bacteria. Their resistance to physical agents such as heat is comparable with that of the acid-fast, typhoid and diphtheria bacilli. *Brucella* are destroyed in milk by exposure to 142° F. for 20 to 30 minutes even when the milk is heavily inoculated with the organisms. Organic materials afford much protection against toxic and injurious agents and procedures. Thus, it has been claimed that pastures contaminated by the *brucella* present in dead fetuses and fetal membranes remain infective for as long as six months. Quantitative studies on the survival of *Brucella suis* suspensions in the presence of dextrin or of ascorbic acid-gelatin mixtures revealed 35 per cent of the organisms to be still viable and fully virulent after six months at 25° C. When collected from aerosols onto cotton impingers, 45 per cent of the nebulized organisms remained viable for 24 hours at 12 per cent relative humidity.

During the aging process in cheese production, exposure to the acidity resulting from the lactic acid fermentation kills within a few days the *brucella* present in contaminated milk.

### DYE TOLERANCE

The species of *brucella* are characterized by certain dye tolerances useful in differentiation. These are determined by adding thionin and pyronin to semisolid agar media and inoculating with about 1,000 organisms. The presence or absence of growth is read in 72 hours. The results obtained by Huddleson, and amply confirmed by others, reveal that, in general, *melitensis* varieties

PLATE 2



Guinea pig 30 days after infection with approximately 50 *Br. suis* cells by subcutaneous inoculation. Spleen is exposed to reveal characteristic appearance of *suis* infections in guinea pig.





grow on both thionin agar (1:50,000) and pyronin agar (1:200,000), while *Br. abortus* grows only on the pyronin and *Br. suis* only on the thionin agar. It must be recognized, however, that each species contains strains which gradually merge into the other species with respect to dye tolerances.

### DISSOCIATION

Avirulent strains spontaneously agglutinable in saline and in 1:1,000 trypanflavine, and inagglutinable in high titer sera directed against the smooth forms were formerly called "para" strains but are now recognized as rough variants. One can recognize several dissociant colony types under a low-power objective, using oblique reflected light. These types differ in colonial consistency and tinctorial aspects from the normally smooth virulent greenish-blue or gray-blue transparent colony (Henry, 1933). The degree of dissociation is governed by rapidity of transfer, temperature, population density, nutrients, pH, and redox potential. R forms exhibit higher viability and slower growth rates than S forms and, therefore, gain ascendancy in cultures unless the medium contains a substance found in the serum or plasma of cows, hogs and goats which inhibits them (Braun, 1946).

### IMMUNOCHEMICAL CHARACTERISTICS

Although *Brucella* species cannot be differentiated by simple agglutination tests, the agglutinin absorption reactions reveal the existence of two main antigenic groups: one including the *abortus* and *suis* species and the other the *melitensis* species. It has been postulated that the brucella contain two antigens, A and M, in different quantitative proportions, and there is evidence that the distribution of A and M on the cellular surface of the *abortus* species is in the ratio of about 20:1 (Miles, 1939). Although attempts to separate the A and M

antigens have so far failed, there is evidence that the two antigens, while closely related, are not identical (Pennell and Huddleson, 1938). The present system of species classification, based on the sulfide production and dye tolerance, is not always in agreement with the serologic grouping, since certain *abortus* strains (biochemical identity) may fall into *melitensis* antigenic groups (Veazie and Meyer, 1936). The picture of species gradation obtained from the biochemical and dye tolerance tests is also given by the results of serologic analysis.

Miles and Pirie prepared the "native antigen" of brucella by killing and extracting the agar-grown cells in either 2 per cent phenol or chloroform water for several days at 0° C. In addition to the A and M factors, the native antigen contained R-phase components. The complex antigen could exhaust the agglutinins from antisera prepared against *Br. abortus* and *Br. melitensis* and was capable of inducing skin sensitivity. A phospholipid could be removed from it without loss of serologic activity. An arginine-containing protein could also be removed, leaving the precipitating power in the residual antigen. After removal of the phospholipid and protein, the residual material was similar to the Boivin type antigens isolated from other species of Gram-negative bacilli. It was heat stable and toxic, and was believed to be an acid with an equivalent weight of 8,000, a molecular weight between  $10^5$  and  $10^6$ , and a sedimentation constant of  $50 \times 10^{-13}$ . Acid hydrolysis liberated a second phospholipid, free phosphate, formic acid and free amino groups and destroyed toxicity and serologic activity (Miles and Pirie, 1939).

### RANGE OF PATHOGENICITY

The three species have a fairly broad range of pathogenicity. Moreover, chronic infection and latency occur both in man and in the various naturally infected animals.



*Br. melitensis* is pathogenic for goats and sheep, which appear to be the primary hosts. In these animals abortions occur, although with irregularity, and latent localized infections persist for several years following acute infection. *Br. melitensis* has also been found in natural infection of man, cattle, swine and wild guinea pigs. Infected cattle

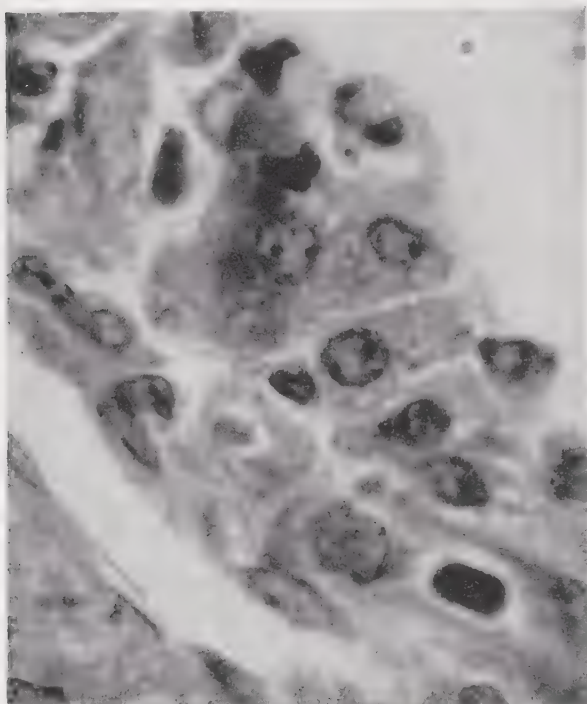


FIG. 24. Parasitized interstitial cells from the testis of a guinea pig inoculated with *Br. abortus*.  $\times 825$ . (Obtained through the courtesy of Dr. M. Ruiz Castaneda, Mexico City.)

transmit the disease to man through milk; swine have been incriminated as the source of an outbreak of *melitensis* infection in Iowa (Jordan and Borts, 1946). It is now generally believed that *Br. melitensis* is more infective for man than either *Br. suis* or *Br. abortus*, but little information is available concerning the incidence of latent infection, or the type of mild chronic infections so often observed in *Br. suis*.

*Br. abortus* has been isolated from naturally infected horses, goats, dogs, sheep, fowl, guinea pigs, wild rats and man, but swine are relatively insusceptible to it. In

the cow, *Br. abortus* is restricted primarily to the pregnant uterus (especially the chorion) and secondarily to the udder ducts. There is no clinical disturbance and no sign of mastitis. Invasion of the chorionic epithelium and multiplication therein is characteristic of *Br. abortus* infection. The bacterial invasion passes from the chorion to the cotyledon, blocking the vascular villi and the fetal circulation. The organisms which escape into the udder from the circulation gain access to the milk and are probably retained in the residual milk and multiply in it. Hence, the disease in cattle is chronic, with the infection equilibrated between the fetal membranes and the udder.

*Br. suis* primarily infects swine but may also be found in natural infections of man, cattle, horses, dogs and fowl. The Danish strains are of low virulence for cattle and man, no human cases having been reported. The American strains, however, are extremely pathogenic for man and may well match the infectivity of *Br. melitensis*. While the organism causes abortion in sows, this is less frequent than the occurrence of persistent localized lesions, for example, in the testes or skeletal system.

Experimental brucellosis can be established in the guinea pig, rabbit, mouse, monkey, cotton rat and hamster. The guinea pig is considered to be the most susceptible animal, although inoculation usually leads to a chronic infection, with recovery if acutely fatal doses are not employed. Differentiation of species by laboratory animal inoculation is not possible.

The chief characteristics of brucellosis in the guinea pig have been thoroughly described (Smith and Fabyan, 1912). After 4 or 5 weeks the spleen may be enlarged, hyperemic, and show many minute foci which are gray and firm. Subcutaneous lymph nodes are swollen. The kidneys become pale and reveal coalescing foci in the cortex. The epididymus is the chief site of testicular lesions. This focus becomes infiltrated and filled with polymorphonuclear

cells and presents the appearance of a cheesy mass. The liver often shows small grayish foci. No lesions occur at the site of inoculation unless a very massive dose has been inoculated subcutaneously, under which circumstances a large abscess may form, involving the subcutaneous musculature and adjacent tissues. Death rarely occurs unless massive doses are injected, and then only after a protracted infection period of several weeks. Intracerebral incubation of moderate doses leads to acute septicemia and death in a few days. The organisms can be recovered from the spleen, lymph nodes and bone marrow. Cultures from the liver are often sterile. In an old chronic infection of the guinea pig, all but the lymph nodes may be sterile.

### PATHOGENESIS

The pathways of infection accepted as the most frequent are the intestinal and percutaneous. However, the ease with which animals and probably humans can be infected by the respiratory route through the use of aerosols demands cautious analysis of the portal of entry for any given case. The organisms entering through the mouth may gain entrance to the tissues via the mesenteric lymph nodes or the lymphatic system in the throat. Experimental oral infection in animals is generalized very rapidly. Organisms disappear from the blood and the liver within 24 to 48 hours and one week respectively but may persist for several weeks in the axillary and inguinal lymph nodes and in the spleen. Bile has been found to harbor the organisms sufficiently often to suggest that it is a site of prolonged infection and cellular multiplication. Intravenous inoculation of rabbits and guinea pigs leads to prompt appearance of the brucella in the bile. The hematohepatic route of infection is the usual one in animals and probably in man.

The nodular reactions found in the lymph nodes, liver, spleen, bone marrow and else-

where in human brucellosis correspond to the response of the reticulo-endothelial system to the phagocytosis which occurs in the vicinity of bacterial proliferation. Monocytes, histiocytes and lymphocytes constitute the predominating cell types. Whatever the mechanism of their formation, these nodules appear to account in part for the latency and chronicity of infection. In acute cases, the renal pathology may be due to the elimination of *Brucella* through the glomerulus and to the intracytoplasmic growth of the organisms in the epithelium of Bowman's capsules and the proximal end of the convoluted tubules (Meyer, 1943).

During infection, the organism leads an intracytoplasmic existence in the mesenchymal and ectodermal tissues. Castaneda followed the organisms through the stages of the acute experimental infection and established their presence within macrophages, fibroblasts, endothelial and reticular cells, interstitial cells of the testes, and alveolar cells.

As in the case of malaria, spirochetosis and endocarditis, repeated showerings of bacteria from the infected foci into the blood stream appear to account for the bouts of fever. However, repeatedly negative blood cultures at this stage have suggested that additional factors including autolysis of host cells, disintegration of *Brucella* in the phagocytes, and formation of the antibody may play a role in the genesis of the fever. The symptomatology of infection has also been considered, without convincing evidence, to be the expression of an allergic inflammatory response. It is quite possible that the constant discharge of antigenic material may be responsible for the general and local reactions observed in subacute and chronic infection.

### PATHOLOGY

It is generally agreed that the reticulo-endothelial system is the site of major changes and that the lesions can be char-



acterized as infectious granulomata. The great variety of symptoms emphasizes the general nature of the infection and the occasional suppurative lesions in various organs illustrate the localizing tendency. Three types of fatal brucellosis have been described: (1) the septicemic or relatively acute form, (2) the focal or localized form, also relatively acute, and (3) the chronic lymphogranulomatous type with prolonged course.

Histologically the basic reaction of brucellosis is a progressive proliferation of the large mononuclear cells of the reticulo-endothelial system, with fibrin exudation and sometimes hemorrhage. Coagulative necrosis and fibroblast proliferation occur later. Plasma cells, lymphoid cells and eosinophilic polymorphonuclear leukocytes also take part in the basic reaction (Albertini and Lieberherr, 1937).

### IMMUNITY

The criteria which have been used to evaluate the immune state have been primarily the ability of the animal host to rid its tissues of the organisms and the control of abortion and of the shedding of *Brucella* in the milk.

In a general way, natural immunity to brucellosis appears to be inversely related to the age of the animal. Up to a certain age calves possess a high degree of resistance which is not due to the ingestion of colostrum-containing antibodies (Huddleson, 1943). This refractory state disappears when the stock is still young. The animals become most susceptible during pregnancy, and again become relatively resistant during the nonpregnant periods. Similarly the incidence of manifest human infections in the age group under ten is low, and it is remarkable how seldom children who drink large amounts of milk show signs of infection. The fact that the sera of children have not been examined so thoroughly as adult sera and that the disease may remain un-

recognized accounts for only a part of the different age incidence. It has been suggested that the consumption of heated milk by children may explain the lower incidence of infection in this age group. The important implications of the "serum selective factor" in this regard are of prime interest, for as Braun (1946) has shown, the appearance and disappearance of this S→R controlling factor is well-correlated with the susceptible state.

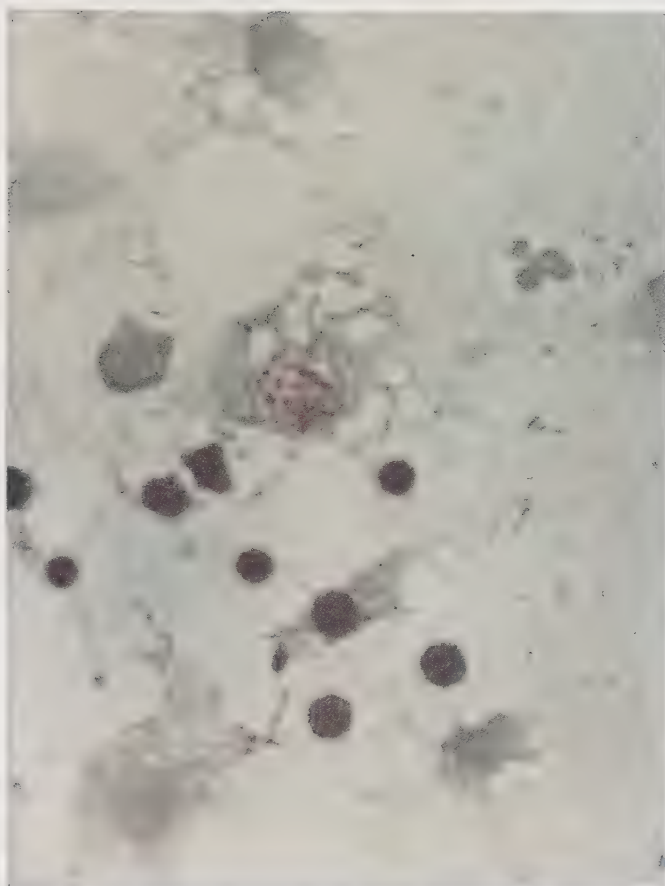
The production of artificial immunity in animals has been studied chiefly with vaccines consisting of living brucella of reduced virulence. In most common use today are two strains, S19 and Strain 45(20) of *Br. abortus*.

Vaccination with the S19 strain produced greater resistance in animals which were adolescent or mature at the time of vaccination (from 7 to 12 months old and even older) than in calves vaccinated when younger than nine months (Haring and Traum, 1943). It must be emphasized that the agglutinins which appear as a result of vaccination cannot be distinguished from those which follow natural infection and that this similarity renders more difficult or prevents the serologic diagnosis of the disease. However, this difficulty in diagnosis is decreased by the fact that the age of the animal on which vaccination is practiced has much to do with the persistence of the agglutinin titer (Haring and Traum).

The avirulence of the strains used for vaccination is only relative as shown by reports of abortion induced in pregnant animals and of cases of undulant fever in human beings caused by these strains.

The main purpose of vaccination of cattle is to delay the development and allay the effect of the infection rather than to prevent it. Depending upon the age of the animal at vaccination and the size of the challenge dose, the immunized animal can develop immunity to abortion, or to localization in the udder or can even sterilize its tissues.

PLATE 3



*Brucella suis* in 9-day-old chick-embryo yolk sac, stained by Macchiavello's method and illustrating intracellular and extracellular forms.





The beneficial effect of vaccination (using *Br. abortus* strain 45/20) of serologically negative animals living in an infected environment was most clearly recognized in the second and later pregnancies following vaccination. Apparent breakdowns in immunity, particularly during the first pregnancy after vaccination, were ascribed to the vaccination of infected nonreacting animals. Many years of study have led to the view that higher resistance to brucellosis is obtained by vaccinating adult cows (up to fifth month of pregnancy) than by vaccinating calves.

### CLINICAL PICTURE

The average natural incubation period in man varies from 10 to 30 days. The onset is ill defined (fatigue, weakness, headache and backache). Fever of 102° to 105° F. may be reached slowly. Arthritis and neuritis occur at any stage of the infection. As the temperature drops periodically, chills and drenching perspiration occur. In the United States febrile relapses are not common, the usual case showing one long febrile period returning to normal by lysis (Simpson, 1941). Anorexia and constipation are the chief gastro-intestinal symptoms and a retention jaundice is often overlooked. This stage with many variations in the acute phase may progress to a chronic stage of the disease for which over 150 different symptoms have been catalogued. During the chronic stage central nervous system lesions may reveal themselves in personality changes and other signs often grouped under the term neurobrucellosis. The symptoms may last several months or subside within a very short time, although complete recovery is difficult to achieve. In view of the fact that cutaneous sensitivity and agglutinating and opsonizing antibodies occur in individuals who offer no history of an attack of the disease, it is likely that latent unrecognized infections are common.

### DIAGNOSIS

Four laboratory procedures are used for the diagnosis of brucellosis: (1) isolation of the causative agent; (2) agglutination test; (3) bactericidal test; (4) opsonocytaphagic test. Isolation of the causative organism is the only convincing evidence of infection. The organisms exist in the blood in relatively small numbers, probably because they occur primarily in the white blood cells. No definite relation has been established between any given stage of the disease and the presence of organisms in the blood; their presence has been noted as early as the first day of illness and as late as the ninth or tenth month. It is very difficult to isolate organisms at any time from the blood of chronic cases. They are excreted also irregularly in the urine and in the stools. They can be recovered from the spinal fluid and pus in cases of meningeal involvement or in suppurative lesions. They have also been isolated from blood clots originally submitted for Widal and Wassermann tests, from bone marrow and bile.

When milk or cream samples are involved, injection of an aliquot of the centrifuged skim milk and of the gravity cream subcutaneously into guinea pigs should be performed simultaneously with the preparation of cultures on agar.

Agglutinin titers of 1:80 or more occur in about 90 per cent of brucella infections by the end of the second week and may eventually reach 1:10,240. The titer may show an irregular course, especially in chronic cases. Heat-killed, phenol-preserved suspensions made from a large representative group of absolutely smooth strains of the 3 species are required for the preparation of agglutinating antigens. The tests should be incubated for 48 hours at 37° C. The presence of broad inhibition zones may cause difficulty with some sera. Agglutination titers of 1:40 or over in the presence of clinical symptoms suggestive of brucellosis constitute presumptive evidence of active infec-



tion. In the absence of symptoms, these titers may be evidence of frequent exposure, e.g., occupationally or through the consumption of infected milk. A titer of 1:40 or less in the absence of clinical signs may be evidence of a latent infection or of a past infection which may have remained latent. Many reports of very low titers or completely negative agglutination tests in active cases of brucellosis indicate that even completely negative agglutination tests may not exclude the possibility of infection. In this connection, large prozones and "blocking type" antibodies must be considered.

Precipitins and complement-fixing antibodies are present in most infections. The complement-fixation test in humans may be positive earlier than the agglutination test and may outlast it. The precipitin test using filtrates of old broth cultures agrees fairly well with other tests but offers no advantage diagnostically.

The use of the bactericidal test in the laboratory diagnosis of active brucellosis has acquired a fresh impetus as a result of recent investigations. The plasma of normal animals inhibits the growth of brucella, whereas the plasma of infected animals does not. Complement is necessary for the antibacterial action, and there appears to be a prozone with excess of serum.

The opsonocytophagic test designed to measure the ability of polymorphonuclear neutrophils to phagocytize brucella, has also been subjected to extensive evaluation, but opinions on its usefulness vary. In combination with the skin test the opsonocytophagic test may have some value as an aid in prognosis. Huddleson and his associates observed that the leukocytes in the blood of patients recovered from brucellosis phagocytized the organisms under certain carefully defined conditions. Leukocytes taken from the blood during infection and from the blood of uninfected persons showed less phagocytic activity. Interpretation of this

test will be considered following the section on skin tests.

The intradermal test, which should be performed only after serum for the agglutination and opsonocytophagic tests has been obtained, involves a technic and interpretation comparable to that of the tuberculin test. Skin sensitivity is acquired through infection. Although it may not appear early in the course of the disease, it persists and is, therefore, of considerable help in the chronic infection. The reagents primarily in use today include "brucellergin," which is a nucleoprotein extract, "Melitin" or "Abortin," which are filtrates of old broth cultures, and suspensions of heat-killed *Brucella abortus*. Each preparation has its proponents but "brucellergin" (Huddleson, 1943) and the cell suspensions are the most widely used. One-tenth cc. of a 1:2,000 dilution of "brucellergin" is injected intradermally, and degrees of erythema, edema and induration are read 48 hours later. Reaction to the commercially available cell suspensions occurs in 12 to 24 hours and appears to be more specific. Reactions to the skin test in sensitized persons often are severe both locally and constitutionally.

The results of skin test, agglutination test and opsonocytophagic test have been evaluated as follows (Huddleson, 1943). No reaction in the 3 tests represents a susceptible individual. A positive skin test with from only 0 to 40 per cent of cells participating extensively in the opsonic test in the presence of either a positive or negative agglutination indicates an individual who has been or is infected, but who has developed little resistance. In the presence of suggestive symptoms a tentative diagnosis is justifiable. In the presence of either a positive or negative agglutination test, a positive skin test with 60 to 100 per cent of the leukocytes markedly active suggests a relatively immune state which can be overcome by infection. An immune state may also exist in the absence of skin allergy.

## TREATMENT

The comprehensive review published in 1936 by Carpenter and Boak almost reflects the present state of the problem. The frequent remission of the infection and the difficulty of diagnosing the chronic form makes an evaluation of any therapeutic procedure difficult unless at least several years of the patient's history, coincidental with and subsequent to treatment, is available.

Many vaccine preparations have been tried with varying success since Wright first used a heat-killed *Br. melitensis* suspension. The results which have been obtained are primarily due to the generalized systemic reaction induced by these preparations and appear to be independent of the type of vaccine used. At the present moment the position seems to be that selected chronic cases do respond to vaccine therapy when the general reaction is kept to a minimum using the subcutaneous route.

The toxic filtrates known under the names of "melitin" and "brucellin" consist of the bacteria-free filtrates of old broth cultures. Brucellin affects the course of the disease by producing a systemic allergic reaction which, in turn, is accompanied by a neutrophilic leukocytosis and increase in immune opsonins. The efficacy of the agent depends upon the existence and continuation of a state of sensitization in the patient while under treatment (Huddleson, 1943). Brucellin appears to work best on early diagnosed cases; its usefulness in chronic cases is still under test. While some of the effects may be due to nonspecific shock, the requirement of a state of sensitization for successful treatment points to a more specific action, as in the case of the vaccines mentioned above. Again as above, there is need for further evaluation of the therapy on large numbers of chronic cases.

It is impossible at the present time to establish any basis for serum therapy in view of the confusing serologic picture in

infected animals and humans. There is no obvious relation between concentrations of circulating antibody and the course of the infection. Furthermore, the behavior of the serum of infected persons in bactericidal tests suggests that the antibody may be present in excess—hence, ineffective as a therapeutic agent. Others consider that the antibody is incomplete in its structure at certain stages of the infection and hence cannot destroy the organisms although it may combine with them.

Sulfonamide derivatives appear to affect primarily the extracellular organisms, thereby relieving those symptoms due to them.

The usefulness of the newer antibiotic substances is still in process of evaluation. While tests in vitro and in chick embryo show that streptomycin can inhibit the growth of brucella, five series of therapeutic trials reported to the time of this writing indicate that the drug alone has little value in the therapy of brucellosis, except perhaps in those cases where the treatment is started very early and is very intensive.

However, a most promising synergistic phenomenon between sulfadiazine and streptomycin has been reported and gives evidence of providing the most efficient therapy. The therapeutic regime consists of 0.5 gram streptomycin every 6 hours for seven days simultaneously with sulfadiazine given for two to three weeks, 1 gram every 4 hours (Spink et al., 1948).

## EPIDEMIOLOGY

Man becomes infected with brucella either through contact with, or ingestion of, infected animals and their products. The response to infection is characteristic of the host and not of the infective strain. The major transmission pathways today for man are via infected milk and milk products and by direct contact with the infection reservoir. Portals of infection are



primarily the alimentary tract and through the skin; the importance of the parenteral routes, such as respiratory, appears greater than is generally recognized.

Marked differences in the incidence of brucellosis exist between different countries and have been ascribed to peculiarities in the virulence of the type of brucella prevalent; as yet, however, no reliable data on comparative virulence are available. In the United States the number of reported cases for the period from 1940 to 1946 have steadily increased from 3,310 to 5,049 per year, except for a slight drop in 1942. In all, 27,299 cases were reported, with 547 deaths during the period. The annual incidence of chronic brucellosis in the United States is very much higher.

The incidence of the bovine disease in the United States is indicated by a recent summary covering the period July 1, 1934, to June 30, 1946. Of 75,247,842 cattle tested by means of the agglutination reaction in the Federal-State co-operative control program, 3,255,185 were judged as reactors (complete agglutination at 1:100).

In humans the incidence in males is considerably higher than in females in farm groups and in those in contact with the livestock industry. However, the incidence in the two sexes is more uniform in other groups, indicating that the difference is not due to sex susceptibility but to the extent of exposure (Hardy et al., 1931). Occupational incidence has been studied among veterinarians, slaughterhouse workers, meat packers, cooks, butchers, milkmen and dairymen, laboratory workers and animal caretakers. The results of the surveys in several countries appear to warrant the following interpretation. Individuals who have no contact with the organism via milk, milk

products or other infected material do not possess cutaneous sensitivity, agglutinins or opsonocytophagic capacity. Among the groups tested above who come into contact with brucella the skin and opsonic tests reveal infection according to the occupation, materials handled and environment (Molinelli, 1939). The number of reactors appears to be in direct relation to the length of time of exposure. The largest number of the occupationally exposed give evidence of latent infection. In a small percentage of infected persons temporary indefinite clinical disturbances were noted.

### CONTROL MEASURES

Because of the multiple sources of infection which maintain the human disease, the problem of prevention is no longer simply concerned with the eradication of bovine brucellosis and the pasteurization of milk. Pasteurization of milk and cream, however, effectively breaks the chain of infections due to ingestion of contaminated milk and remains at the present time the only procedure available to minimize ingestion of the bovine type of brucella by humans. It is hoped that active immunization of animals, and the segregation of those suffering from brucellosis, will contribute to decrease human contact infection. The organisms leave the infected host via the urine, stools and sputa. In view of their persistence in these organic materials, strict disinfection procedures should be used as in the case of enteric diseases. No terminal disinfection measures are required in the human disease, but in animal brucellosis, disinfection of the afterbirth is essential to prevent spread of the disease through the herds.

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## 21

# Listeria and Erysipelothrix

### INTRODUCTION

*Listeria* and *Erysipelothrix* are Gram-positive, rod-shaped, non-spore-forming organisms which are pathogenic for a wide variety of animals. Although the disease picture produced in man by these two groups of organisms is dissimilar, the two groups are related from a bacteriologic standpoint, and some workers place them in a single genus.

### LISTERIA MONOCYTOGENES

#### HISTORY

*Listeria monocytogenes* is a causative agent of sporadic cases of meningitis in man and also has been cultured occasionally from the blood stream of patients with an infectious mononucleosislike syndrome. The organism was first isolated by Murray et al. (1926) from rabbits and was described under the name *Bacterium monocytogenes* in view of the mononuclear leukocytosis which occurred in these animals. Pirie created the generic name *Listerella* for an identical organism obtained from an epizootic in wild rodents; this proved to be a homonym, and subsequently the name *Listeria* was proposed (Pirie, 1940).

#### MORPHOLOGY, CULTIVATION AND BIOLOGIC PROPERTIES

*Listeria monocytogenes* occurs in both a smooth and a rough form. In the smooth phase, small rods predominate; these

measure  $0.5\ \mu$  in width by 1 to  $2.5\ \mu$  in length and occur singly or in short chains. In the rough phase, young cultures consist almost entirely of long filaments averaging  $60\ \mu$  in length, while older cultures contain short filaments, rods, and a few coccal forms. It is sluggishly motile with a characteristic "tumbling" or spiral movement; cultures grown at  $37^{\circ}\text{C}$ . contain nonflagellated and monotrichous forms, while at room temperature the organism is peritrichous with a maximum of four flagellae. It is Gram positive, non-spore-forming, and facultatively anaerobic.

Growth on the simpler media is scanty to moderate in amount, with improvement resulting from the addition of glucose, ascitic fluid or defibrinated blood. In the smooth phase on nutrient agar after 24 hours incubation at  $37^{\circ}\text{C}$ . the colonies measure up to 0.8 mm. in diameter and have a smooth surface; they are almost transparent by transmitted light. Colonies in the rough phase are slightly larger and develop a granular center. In broth the smooth form produces a diffuse turbidity, while the rough form gives a threadlike granular growth. On horse, rabbit or human-blood-agar plates, nearly all strains produce a narrow zone of beta-hemolysis.

*Listeria* produces acid without gas in glucose, levulose, maltose, mannose, rhamnose, trehalose, dextrin and salicin; variable results are obtained with glycerol, lactose,

sucrose and starch. Gelatin is not liquefied, and H<sub>2</sub>S and indole are not formed (Seastone, 1935; Barber, 1939; Harvey and Faber, 1941; Julianelle, 1941a; Griffin and Robbins, 1944). Growth develops in casein or gelatin hydrolysate media containing glucose and inorganic salts when riboflavin, biotin and hemin are added (Hutner, 1942).

The antigenic structure of *Listeria monocytogenes* has been extensively studied by the agglutination and agglutinin absorption technic. Paterson (1940) and Robbins and Griffin (1945) recognized 4 types each containing one type-specific and at least one species-specific factor. The species-specific factor of the flagellar antigens caused cross agglutination to high titer among all strains studied. Recently Drew (1946) divided available strains of *Listeria* into two serologic groups on the basis of their cellular polysaccharides.

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

Infections in man or animals have been reported from many localities, indicating that *Listeria* has a world-wide distribution. Various hosts are susceptible to spontaneous infection including the rabbit, guinea pig, gerbille, fox, cow, sheep, goat and chicken. The common laboratory animals may be infected experimentally; mice are extremely susceptible, and monkeys relatively resistant.

#### PATHOGENESIS AND SYMPTOMATOLOGY

In man and the ruminants *Listeria* may produce a meningoencephalitis, while in smaller animals there is a generalized septicemia with focal abscesses in the liver and often in the myocardium. The infection in rabbits, guinea pigs and chickens is usually accompanied by a monocytosis; this has not been observed in sheep or cattle. In rabbits this mononuclear response has been intensively studied by Conway (1939) who

concluded that the cells arose from lymphoid tissue.

Approximately 20 cases of *Listeria* meningitis in man have been reported from various sections of the world. Clinically the picture resembles that of the other purulent meningitides or else of an encephalitis. Of the 4 fatal cases described by Burn, 3 had positive blood cultures and all showed small focal areas of necrosis in the liver (Burn, 1936). The type of cellular response in the spinal fluid has been variably reported as being mononuclear or polymorphonuclear in nature.

Interest developed regarding the possible etiologic role of *Listeria* in infectious mononucleosis following the report of Nyfeldt (1929). Subsequently he was able to isolate *Listeria* on nine occasions from the blood or spinal fluid of patients with an infectious mononucleosislike entity. Pons and Julianelle (1939) and Webb (1943) each reported a single isolation; however, these and other workers frequently have failed to obtain positive cultures. Serum specimens from cases contain agglutinating antibodies in low titer against *Listeria*, with no demonstrable rise in antibody titer occurring during convalescence; however, this finding is of questionable significance for in known human *Listeria* infections low-agglutinin titers have been found. Kolmer (1939) found that rabbits immunized with *Listeria* failed to develop heterophile antibodies. At present no conclusive answer is available regarding the possible etiologic role of this organism. The problem is reviewed by Julianelle (1940), Janeway and Dammin (1941) and Webb (1943).

#### DIAGNOSIS

The diagnosis of human infection rests on the isolation of the organism from blood or cerebrospinal fluid cultures. In a study of an outbreak of encephalitis in sheep, Biester and Schwarte (1939) had difficulty in isolating *Listeria* unless brain emulsion



was used as the inoculum; a similar technic might be applicable in fatal human cases. Care must be taken that these organisms are not confused with beta hemolytic streptococci or else discarded as diphtheroids. In the specific identification, the reaction produced in the rabbit eye is of value; *Listeria* instilled into the conjunctival sac produce a conjunctivitis and corneal involvement (Julianelle, 1941a).

#### SPECIFIC THERAPY

Both clinical and experimental evidence suggest that sulfonamides are of value in the treatment of human infections (Porter and Hale, 1939; Savino, 1940). *Listeria* strains, on the other hand, appear to be relatively resistant to the action of penicillin (Foley et al., 1944; Handelsman et al., 1946).

#### EPIDEMIOLOGY

The mechanism of infection in man is not known. It is perhaps suggestive that susceptible animals such as mice and sheep can be experimentally infected by the intranasal route.

### ERYSIPELOTHRIX RHUSIOPATHIAE

#### HISTORY

*Erysipelothrix rhusiopathiae* is the cause of erysipeloid, a relatively common cutaneous infection in man. Erysipeloid must not be confused with human erysipelas of streptococcal origin. Further confusion may arise from the fact that *Erysipelothrix* infection in swine is referred to as "swine erysipelas."

Organisms of the *Erysipelothrix* group were isolated by Koch in 1880 from mice and by Loeffler in 1886 from swine. Rosenbach was the first to apply the term erysipeloid to the disease, and in 1886 he isolated the etiologic agent (Rosenbach, 1909). It is now generally held that strains of

human, porcine and murine origin are almost identical variants of the single species, *E. rhusiopathiae*. Recently the similarities between *Erysipelothrix rhusiopathiae* and *Listeria monocytogenes* have been pointed out, and both organisms have been placed in the genus *Erysipelothrix* (Topley and Wilson, 1946). However, differences in motility and pathogenicity, and the lack of an antigenic relationship between the two organisms may delay general acceptance of this proposal.

#### MORPHOLOGY, CULTIVATION AND BIOLOGIC PROPERTIES

Morphologically, *E. rhusiopathiae* is similar to *Listeria*, occurring in a smooth and rough form, but differs in being nonmotile. It is Gram positive, non-spore-forming, and no capsule has been demonstrated. In the smooth phase, the organisms are slender rods measuring up to  $0.4\ \mu$  in width and  $2.0\ \mu$  in length. In the rough phase there are long chains of bacilli and filaments of variable length.

Growth on simple media is scanty in amount, but is improved by the addition of glucose and serum. The smooth phase grows better at  $30^{\circ}\text{C.}$ , while at  $37^{\circ}\text{C.}$  growth of the rough variant is favored. In the smooth phase, round, glistening, water-clear colonies 0.1 mm. in diameter are produced on nutrient agar after 24 hours incubation at  $37^{\circ}\text{C.}$  In the rough phase, the colonies are larger with a granular appearance and a fimbriate edge. The formation of lateral outgrowths in gelatin-stab cultures giving rise to a "lamp-brush" appearance is a diagnostic feature but is not a constant finding; gelatin is not liquefied. On blood agar most strains produce partial or alpha hemolysis around deep colonies. *E. rhusiopathiae* is microaerophilic. The sugar reactions are variable, but typically acid without gas is formed in glucose, lactose and levulose (Barber, 1939; Watts, 1940; Julianelle, 1941b; and Hutner, 1942).

Watts (1940) recognized two distinct antigenic types of *E. rhusiopathiae*, whereas later Gledhill (1945) differentiated four groups, but found numerous unclassifiable strains and concluded that the various strains were qualitatively homogeneous with a large number of different antigens, but that they differed in the quantitative distribution of the antigens.

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

*Erysipelothrix rhusiopathiae* has a world-wide distribution. Natural infections have been reported in a wide range of animals, including man, swine, sheep, mice, cattle, horses and domestic fowl. It can survive for months in decomposing nitrogenous material and may occur as a soil saprophyte. Mice and pigeons are highly susceptible to experimental infection, while guinea pigs are resistant. Fish fed on infected meat develop an asymptomatic type of infection.

#### PATHOGENESIS AND SYMPTOMATOLOGY

While *Erysipelothrix*, like *Listeria*, may produce a generalized septicemia with focal liver lesions in susceptible animals, it differs in manifesting a predilection for the skin, endocardium and joints. In man, the disease usually takes the form of a localized cutaneous infection, although rarely septicemia with endocarditis and joint involvement may occur (Klauder et al., 1943). In swine, 3 forms of the disease are common: a mild form known as "diamond skin" disease, in which skin involvement predominates, an acute severe form with symptoms of septicemia, and a chronic form characterized by arthritic symptoms.

Interest in erysipeloid in the United States dates from the report of Gilchrist in 1904 in which 329 cases seen in Baltimore were recorded; 323 cases were in individuals who had suffered abrasions while handling crabs. Characteristically the infec-

tion develops following an abrasion incurred while handling organic matter, especially fish, shellfish, meat or poultry (Klauder, 1938). From two to seven days later pain occurs at the site of inoculation and is followed by edema and erythema, which is of a characteristic purplish red-color. The margin of the erythematous zone is sharply defined and slightly elevated; as the lesion develops there is peripheral extension with clearing of the central portion. Relapses are common and new lesions may appear at remote sites. Pain and malaise are the chief subjective symptoms. Fever is usually not present. One attack does not confer complete immunity, and reports of reinfections are common.

#### DIAGNOSIS

The specific diagnosis of erysipeloid rests on the isolation of *E. rhusiopathiae*. Positive cultures are rarely obtained from material collected on swabs from the local lesion. Barber et al. (1946) recommend biopsy of the lesion and culture of the skin fragment for 24 hours in glucose broth, followed by subculture to blood agar plates. *E. rhusiopathiae* may also be isolated in pure culture from the heart's blood of white mice following intraperitoneal injection of a suspension of material obtained at biopsy. In suspected cases of septicemia repeated blood cultures should be performed. No information is available regarding the possible diagnostic value of agglutination tests in man; in swine, agglutination tests have been used in the diagnosis of the chronic forms of infection.

#### TREATMENT

Evaluation of specific chemotherapeutic agents in the treatment of erysipeloid is complicated by the fact that the disease is usually self-limited and runs a variable course although the average duration is about three weeks. Sulfonamide compounds are of doubtful value (Porter and Hale,



1939; Klauder and Rule, 1944). Both experimental and clinical results suggest the use of penicillin in the treatment of erysipeloid (Heilman and Herrell, 1944; Barber et al., 1946). In cases associated with septicemia, administration of immune serum should be considered (Klauder et al., 1943).

#### EPIDEMIOLOGY

Erysipeloid is primarily an occupational disease and is seen in individuals handling

meat, fish, poultry or shellfish. King (1946) summarized 115 cases treated at a London hospital and found that 85 of these were directly attributable to an animal source. In a group of 100 cases studied by Klauder (1938) abattoir workers and fish handlers predominated. Outbreaks of the disease have been described in workers in a bone button factory (Lawson and Stinnett, 1933) and in veterinary students who became infected while dissecting a horse (Morrill, 1939).

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# The Cholera Vibrios

### INTRODUCTION

The *vibrios* are curved rods possessing a single polar flagellum. They are highly motile, non-spore-forming, Gram negative and facultatively aerobic.

*Vibrio cholerae* is the specific cause of the disease cholera in man and does not infect other hosts in nature. It is nonhemolytic, usually ferments sucrose and mannose but not arabinose, grows at higher pH than other enteric pathogens and possesses a specific group somatic antigen. (Synonym: *V. comma*.)

### HISTORY

Long before bacteria were discovered, the features of cholera epidemics had been thoroughly explored and the existence of minute living causative organisms postulated. In 1854, Snow, through masterly detective work in the Broad Street Pump epidemic in London, incriminated a contaminated water source. This is a landmark in epidemiologic research. The well-known Hamburg-Altona epidemic of 1892 indisputably established the theory of water-borne disease. In 1883, Koch discovered the cholera vibrio which was not immediately accepted as the cause of the disease. Indeed, Pettenkofer, Emmerich and some of their pupils were so sceptical that to prove the harmlessness of the vibrio they drank a pure culture of it. Some of them developed diarrhea and one became seriously ill; but the experiment was not crucial, and it remained for subse-

quent bacteriologic and epidemiologic experience to firmly establish the vibrio as the inciting cause of cholera.

In 1893, Pfeiffer made the important observation that a guinea pig which had recovered from an inoculation with cholera vibrios had acquired the power of lysing vibrios which were subsequently injected into its peritoneal cavity. Organisms undergoing lysis first lost their motility, then became swollen and globular in shape and finally disappeared entirely. Pfeiffer further showed that the same bacteriolytic capacity could be conferred on a normal guinea pig if immune serum from another animal were injected along with the vibrios. In 1895, Bordet succeeded in producing the Pfeiffer phenomenon in vitro. He found that a heat-labile substance, named complement or alexine, present in normal serum, was essential to the lytic action of immune serum. In 1897 the precipitin reaction was discovered when Kraus added filtrates of broth cultures of cholera vibrios and plague bacilli to their specific immune sera.

### MORPHOLOGY AND STAINING

Freshly isolated vibrios are comma shaped, about  $0.3\ \mu$  broad and  $4\ \mu$  long (Fig. 2M); they possess a single polar flagellum and are highly motile. Occasionally two or three vibrios are attached end-

to-end and assume the form of a spirillum. When grown on artificial media for prolonged periods they lose their characteristic comma shape and become straight rods indistinguishable morphologically from other enteric bacilli. They are Gram negative and can be stained by the usual aniline dyes. The flagellum is seen only by means of special staining procedures or with the electron microscope.

### GROWTH REQUIREMENTS AND CULTIVATION

Vibrios, including those causing cholera, can be grown on a medium containing a few mineral salts with asparagin as the sole source of carbon and nitrogen. They grow well on all the usual laboratory media, and prefer a temperature of 37° C. Most abundant growth is obtained in the presence of gaseous oxygen, but some strains will grow anaerobically. They will grow at a high pH (9.0-9.6) but are very sensitive to acid. In the presence of fermentable carbohydrate a culture of vibrios will quickly sterilize itself due to acid production.

The colonies of smooth organisms grown on meat-infusion peptone agar are semi-translucent or opaque, moist and with an entire edge. The surface may be smooth or finely granular. Rough colonies may show an irregular edge and a more granular surface, but often they are indistinguishable from smooth colonies. The colonies of the variant known as rugose possess radical or irregular corrugations of a very striking sort correlated with the secretion by the culture of a gelatinous intercellular substance. The rugose form is entirely unrelated to S→R variation.

### BIOCHEMICAL ACTIVITIES

Although no precise classification of vibrios on the basis of their fermentations has been achieved, the regularity with which

strains from cholera ferment mannose and sucrose but fail to ferment arabinose (Heiberg, 1935) justifies the determination of these biochemical activities in the identification of the cholera vibrios. Cholera vibrios produce indole from tryptophane and reduce nitrate. When concentrated sulfuric acid is added to a culture in which these reactions have occurred, a red pigment is formed. This is the cholera red test. A positive reaction is given by almost all cholera vibrios, but is not limited exclusively to them and hence has little diagnostic significance. In performing the test a peptone must be used which contains adequate tryptophane and nitrate, such as Difco Tryptone. Various other biochemical reactions of the vibrios include the conversion of histidine to histamine, of glutamic acid to succinic acid and ammonia and the liquefaction of gelatine.

Some of the vibrios produce a soluble hemolysin which can be detected by adding 24-hour broth culture to a 3 per cent suspension of washed goat erythrocytes. The reaction is read for hemolysis after two hours' incubation at 37° C. and after standing overnight in the icebox. Sometimes strains which do not form the soluble hemolysin produce a slow, incomplete hemolysis on blood agar plates. This delayed hemolytic action has been attributed to a proteolytic enzyme. *V. cholerae* does not produce the soluble hemolysin, but may cause the delayed type of hemolysis.

### RESISTANCE

Cholera vibrios are highly sensitive to acid, and it is doubtful if they can live more than a few moments in gastric juice which contains free hydrochloric acid. They are killed by exposure to 56° C. for 15 minutes, and they succumb to the usual chemical disinfectants. They are very sensitive to drying, but are not adversely affected by diffuse daylight.



## CHOLERAPHAGE

Many races of bacteriophage active against cholera vibrios have been recovered from water, sewage or the stools of patients. These choleraphages have been used extensively in therapy and in the treatment of sewage and water, with equivocal results. It has been suggested that choleraphage plays an important role in the recovery of patients from the natural disease. When bacteriophage is added to a culture of vibrios, in due time resistant variants appear which may differ from the original strain in colony form, antigenic structure, dissociative state, agglutinability and hemolysin production. The action of a race of phage is directed towards some particular constituent of the bacterial cell. Thus one phage is specifically inhibited by the polysaccharide of the smooth form while others are inhibited by certain lipid constituents.

## ANTIGENIC STRUCTURE AND DISSOCIATION

The vibrios undergo dissociative changes of the S→R type, and in addition show a further degradation to the  $\rho$  form. Alterations in colony form are not necessarily conspicuous in this dissociation, but a modification of antigenic structure occurs, which is discussed below. The rough variants agglutinate spontaneously in 0.85 per cent saline solutions, and are agglutinated by acriflavine.

## THE FLAGELLAR ANTIGEN

The vibrios possess a single flagellar antigen (the H antigen) which is identical serologically in all the true cholera vibrios and in many of the noncholera vibrios. The flagellar H antigen is heat labile, being destroyed by exposure to 100° C. for two hours. Immune sera prepared with heated vaccines contain antibodies for the somatic antigens but not for the H antigen. Sus-

pensions of flagella which have been separated mechanically from the cell bodies can be used to produce pure anti-H sera. Unlike the somatic antigen, the H antigen is not modified in dissociation from the smooth to the rough form, and its antibodies do not appear to be involved in the protection of susceptible animals.

## THE SOMATIC ANTIGENS

Early agglutination studies by Japanese workers showed that vibrios isolated from cholera patients share one common antigen, and that there are, in addition, secondary agglutinogens which can be detected by means of absorbed sera. These secondary antigens made it possible to divide the cholera vibrios into three serologic types, Inaba, Ogawa and Hikojima. Inaba possesses one type antigen (A), Ogawa a different type (B), and Hikojima possesses the type antigens of the other two (AB). Gardner and Venkatraman (1935) found that the vibrios can be divided into six major subgroups on the basis of their somatic antigens. Almost all the vibrios from typical cases of cholera belong to subgroup I. Vibrios of subgroups II to VI were isolated from water or from atypical cases of diarrhea. This classification has been very useful and has been universally adopted. Where X is the somatic antigen of subgroup I, A is the secondary somatic antigen of type Inaba and B is the secondary somatic antigen of type Ogawa, the formulas for cholera vibrios in the smooth state can be written: type Inaba, AX; type Ogawa, BX; and type Hikojima, ABX.

The soluble somatic antigens extracted from smooth cholera vibrios are complex molecules of the "complete antigen" type. They are antigenic, toxic and possess polysaccharide fractions which confer upon them serologic specificity. The union between the somatic antigens (as they exist in the intact cell) and the corresponding antibodies results in agglutination reactions of the

rapid, finely granular type; and bacteriolysis occurs in the presence of complement. When properly extracted from the bacteria, these antigens give well-defined precipitin tests.

Guinea pigs (Pfeiffer, 1893) and mice (Griffitts, 1942) can be protected from lethal doses of cholera organisms by immune serum or vaccines. Chick embryos (Wilson, 1946) can also be protected, if complement is added with the immune serum. The protective mechanism in these instances is undoubtedly immune bacteriolysis. In chick embryos, protection is conferred both by group and by type antibodies.

The first immunochemical work on the vibrios was done by Landsteiner and Levine (1917). They isolated an alcohol-soluble-carbohydrate haptene which reacted in a precipitin test. White (1936) investigated the immunochemistry of the somatic antigens and identified four polysaccharide haptenes, which he named  $C\alpha$ ,  $C\beta$ ,  $C\gamma$  and  $C\delta$ . In the normal smooth state all four polysaccharides are present, and  $C\alpha$  is dominant in agglutination reactions. In the rough state,  $C\alpha$  is lacking, and  $C\beta$  is dominant. In the  $\rho$  state, only  $C\gamma$  and  $C\delta$  are present, and  $C\delta$  is dominant. Serologically specific differences in  $C\alpha$  are responsible for the Japanese types  $C\alpha$  (Inaba) and  $C\alpha$  (Ogawa). Linton (1940) criticized White's work, believing that some of the above polysaccharide fractions were chemical artifacts. He agreed with White that the change from smooth to rough was characterized by a loss of the S polysaccharide, but maintained that the rough strains possessed a new polysaccharide which was not represented, even in a concealed form in smooth strains.

White (1940) described a number of additional antigens of the cholera vibrios: (1) A heat-labile protein antigen detectable by a precipitin reaction but not participating in agglutination reactions, which was found in all vibrios, but not in other organisms;

(2) a heat-stable protein antigen likewise participating in precipitin but not in agglutinin reactions, and present in vibrios of various dissociative states; (3) an alcohol-soluble Q protein, similar to that found in the Salmonellas; and (4) a rugose haptene, the intercellular substance of rugose variants, which reacted in agglutination and precipitin reactions.

Linton (1940) has approached the classification of the vibrios from a novel point of view. He isolated two proteins, differing in optical rotation, and three polysaccharides, differing in the hexose obtained on hydrolysis (glucose, galactose or arabinose). By combination of each of the proteins with each of the polysaccharides he divided the vibrios into six groups, which should not be confused with the six serologic groups of Gardner and Venkatraman. Linton's classification has not been widely used and the significance of his groups in relation to the recognized serologic attributes of various vibrios is not known. Recent unconfirmed work of Burrows et al. (1946) has suggested that the O antigens may be more numerous than hitherto believed.

## TOXINS

There is considerable diversity of opinion about the chemical nature of the toxin of cholera vibrios, although it is generally agreed that it is of the endotoxin type. Preparations isolated by various procedures differ in their chemical composition, the analyses indicating that they are proteins, or phospholipids, or part of the "complete antigen" molecule which may contain protein, lipid and polysaccharide fractions. It is possible that vibrios produce more than one toxin. Active preparations of endotoxin are lethal for mice, rabbits and guinea pigs. Many of them are antigenic when measured by in-vitro methods, but no antitoxin has been prepared which will protect a susceptible animal. Burrows et al. (1944) have reported that their preparation of endotoxin



increases the rate of flow of fluid across whole rabbit and guinea pig intestine used as a membrane.

It has been shown by Burnet and Stone (1947) that filtrates of *V. cholerae* contain enzymes which, in vitro, cause desquamation of the intestinal mucosa of guinea pigs. One of these enzymes has been demonstrated to be a mucinase. Together, they may play a part in the pathogenesis of cholera. Burnet, McCrea and Stone (1946) have also demonstrated an enzyme in cholera filtrates which destroys the receptors of red blood cells for virus particles. It is distinct from the desquamative enzymes and has no apparent pathologic role.

### THE EL TOR VIBRIOS

A strain of vibrio isolated from pilgrims in 1905 at the Tor quarantine station has been shown to differ from the true cholera vibrios only in that it produces a soluble hemolysin. It is apparently capable of producing epidemics of diarrheal disease.

### DISTRIBUTION AND RANGE OF PATHOGENICITY

The cholera vibrios infect only man in nature. Rabbits, guinea pigs and mice can be killed experimentally by large parenteral inoculations. Kcch produced infections in rabbits by feeding the inoculum after paralyzing intestinal motility with opium and neutralizing gastric acidity. In guinea pigs, oral administration may lead to an inapparent infection in which the vibrios multiply in the intestinal tract, but the host does not become ill. Developing chick embryos are highly susceptible to small inocula of vibrios.

The survival of cholera vibrios outside of the host is favored by dampness and low temperatures, but in any case it is not of long duration. The vibrios remain viable in rice-water stool and in river water up to 16 or 17 days, and in sea water as long as

4 days. They have been recovered from septic tank contents up to 5 days, and from a few hours to two weeks after experimental contamination of food. These periods are long enough to allow transfer of infection but cannot account for the persistence of organisms through interepidemic periods.

A natural disease of chickens is caused by *Vibrio metchnikovi*, which closely resembles the cholera vibrio, but which is antigenically distinct from it. Vibriolike organisms have been recovered from exudates of aborting sheep and cattle. It should be mentioned that hog cholera and chicken cholera are not caused by vibrios.

### PATHOGENESIS

Cholera is acquired by the ingestion of cholera vibrios from contaminated water (as is usually the case), from contaminated food, or from direct contact with a cholera victim. The ingested vibrios reach the intestine where bacterial multiplication occurs rapidly. The gut wall is irritated by the toxic action of the vibrios and responds with a profuse outpouring of fluid. This is accompanied by diarrhea and leads to profound dehydration of the patient. The stools lose their normal fecal character and resemble rice water. The vibrios remain localized in the intestinal tract, and bloodstream invasion is unknown. Whether there is a systemic absorption of cholera toxin is a matter of dispute. The clinical picture of cholera is one of dehydration, hemoconcentration, toxemia and finally shock. Death may occur a few hours after the onset of symptoms. In the most rapidly fatal cases there is insufficient time for extensive pathologic change to take place, and little may be seen except the rice-water stools, edema of the gut wall and dehydration of the tissues. In more prolonged cases the mucosa of the small intestine is deeply injected and may show areas of sloughing. Sometimes most of the small bowel is denuded of its epithelial lining. The kidneys may show a

toxic nephrosis. Microscopically the small intestine shows hyperemia and sometimes loss of epithelium, but there is little cellular reaction to the infection. Vibrios are present abundantly on the mucosal or denuded surfaces of the gut, but they do not penetrate the submucosa. There are no distinctive pathologic findings in cholera, and the specific diagnosis must be made bacteriologically.

### ACTIVE IMMUNITY

Immunity following recovery from cholera is of uncertain duration but probably lasts many years. Vaccination was first attempted in 1885 in Spain by Ferran. He used living organisms and encountered so many severe reactions and deaths that the procedure fell into disfavor. More recently, killed organisms have been used. The current vaccine approved by the Allied Armed Forces consists of 8,000,000 organisms per cc. of an Inaba and an Ogawa strain, preserved with phenol. Autolysis often occurs in this vaccine on standing, so that it may appear almost water clear. Two inoculations are given at weekly intervals, and immunity is reputed to last from 6 months to a year. The vaccine is standardized by the mouse-mucin protective test of Griffiths (1942).

### DIAGNOSIS

In the presence of an epidemic the clinical recognition of cholera presents no difficulties. Although there are no pathognomonic signs or symptoms, the suddenness of the onset, the severity of the symptoms and the rapidity of the course distinguish it from other diarrheal diseases. Sporadic and mild cases are more difficult to recognize, and the diagnosis is made by isolating the cholera vibrio from the stools and sometimes also from the vomitus of patients.

The vibrios will grow on any of the media usually employed for the isolation of enteric pathogens, but where cholera is sus-

pected clinically, it is advantageous to use a medium which preferentially encourages vibrionic growth. Such media depend in the main on the ability of vibrios to grow at a pH so high that other organisms likely to be encountered in stools are inhibited. The medium of Vedder and van Dam is one of these.

Boil 1.0 gm. hemoglobin in 20.0 cc. N/5 KOH, obtaining as much solution as possible. Cool under running tap water. Add 120 mg. glycocoll and 80 cc. of melted and cooled peptone agar (peptone 1 gm., NaCl 0.5 gm., agar 3 gm., water 100 cc.). Pour plates.

Colonies which grow out well on this medium are transferred to peptone water or veal infusion broth or to a veal infusion agar slant to obtain a pure culture of the strain for subsequent studies. Identification of the strain as *Vibrio cholerae* involves studying the morphology and motility of wet preparations; Gram-stain characteristics; hemolysis; fermentation reactions; reaction in the cholera red test; and finally serologic group and type when suitable diagnostic antisera are available.

Many attempts have been made to devise a standard method for the preparation of diagnostic sera, and the use of heat-treated vaccine containing both Inaba and Ogawa strains has proved most satisfactory. Absorbed sera are necessary for differentiation of the Japanese types. Occasionally, vibrios recovered from sporadic cases of cholera fail to agglutinate with a potent O subgroup-I antiserum. The reason for this inagglutinability is unknown, but the suggestion has been made that it results from dissociative changes induced by bacteriophage.

### TREATMENT

The treatment of cholera is directed toward the patient's disturbed physiology rather than toward the vibrio, and the parenteral administration of fluids constitutes the most important measure. *V. cholerae* is sensitive to the various sulfonamides and



to streptomycin, but penicillin is of little effect. Where proper symptomatic treatment can be given, the chemotherapeutic and antibiotic agents are of secondary value only. Alone, they are inadequate. There are no specific antisera or antitoxins used in the treatment of cholera.

### EPIDEMIOLOGY

Cholera has been endemic in India for many years. In 1817 it started on the first of a series of epidemic waves which continued irregularly throughout the 19th century. Each pandemic extended farther from the endemic source, until Europe was reached (1830) and finally America (1832). There has been no cholera in America since 1892 or in Europe since 1925, but the 1947 epidemic in Egypt was dangerously close. Epidemics continue to appear with some regularity in Asia. In India there were 337,000 deaths from cholera in 1930, and 216,580 cases in 1944. Recently small epidemics have occurred in China, and the disease seems to have become endemic in Burma, Thailand and French Indo-China. Calcutta is the most heavily and persistently infected port city in the East. The world-wide dissemination of cholera in the early epidemics closely followed shipping lanes, overland trade routes and pilgrim migrations. The disease was carried from place to place by individuals who had mild cases of the disease, who were incubating it or who were carriers. Once cholera appeared in a new locality, its spread was usually by means of contaminated water. The worse the hygienic conditions, the faster was the spread and the shorter the course of the local epidemic.

The carrier state in cholera is usually of short duration, and chronic carriers, such as occur in typhoid fever, are unknown. The convalescent carrier is usually free of vibrios in a few days, almost invariably in a month. In water, the organisms may last two weeks. The survival of cholera vibrios

through interepidemic periods is, therefore, not easy to explain, but it probably depends on sporadic cases in the endemic areas. Epidemic spread is favored by periods of high temperature, high humidity and intermittent rains.

The character of the epidemic does not vary significantly with the serologic type of cholera vibrio present. In some epidemics a single type may be found, in others there may be a mixture of types. In India, Ogawa epidemics are common; in Japan there have been pure Inaba epidemics; and in China, Hikojima has predominated. There are no rigid geographic associations, however, and in a given locality the type may vary from epidemic to epidemic. A recent epidemic in Celebes was caused by El Tor vibrios, and it carried the high mortality associated with true cholera.

### CONTROL MEASURES

Cholera is a disease which occurs only under the most deplorable sanitary conditions. Filtration or treatment of water, proper disposal of excreta and sanitary handling of food makes it impossible for cholera to spread in a community.

Where, for various reasons, adequate sanitation cannot be achieved, mass vaccination has been tried. Unfortunately, it is still impossible to judge its value precisely, although cholera vaccine has been used for more than 50 years. The largest experiments were conducted in the Balkans during World War I and in India in 1944 (Report of The Scientific Advisory Board, Indian Research Fund Association). In the Indian epidemic, 1,180,000 out of a population of 3,000,000 were given a single inoculation during the epidemic. The attack rates were considerably lower in the inoculated group, but case mortality rates were not favorably affected. In this epidemic, as in previous ones, the observations were made under difficult circumstances and the

inoculated and uninoculated groups were not strictly comparable. Further experience under properly controlled conditions is necessary to settle the question. The value of treatment of water by bacteriophage as a control measure is still more uncertain.

Rapid air travel increases the possibility of introducing cholera into North America and Western Europe from the Orient, but it is unlikely that it would become estab-

lished or spread in these areas. The American Public Health Association and the British Ministry of Health recommend as control measures the isolation of patients, disinfection of excreta, quarantine of contacts for 5 days from last exposure, immunization of contacts, bacteriologic detection and isolation of carriers, and rigid sanitary measures with respect to water and food and fly suppression.

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The Hemophilus Group

INTRODUCTION

The Committee on Nomenclature of the American Society of Bacteriologists have classified the organisms listed in Table 39 within the genus *Hemophilus* (Bergey, 1948). These organisms have certain characteristics in common; they are Gram negative, nonmotile, non-spore-bearing, aerobic bacilli and lead a strictly parasitic existence; they possess poorly developed enzyme systems; all of them therefore require enriched media. Their nutritional needs, yet imperfectly defined, display certain differences within the genus which have a limited but useful application in identification. As shown in Table 39, the hemophilic bacteria fall into two distinct groups on the basis of their needs for two growth factors, named X and V. Designation of those in the first column as the influenza bacillus group serves a useful purpose.

There has been objection to including all of these members in the genus *Hemophilus* since the term suggests dependence on blood. However, since the growth of all members

listed in the table is enhanced by blood, a name which indicates nothing more committal than "love of blood" may be regarded as appropriate.

*H. influenzae* and *H. pertussis* are the most important members of this genus. The former is discussed in this chapter; the latter is discussed in Chapter 24.

HEMOPHILUS INFLUENZAE

The influenza bacillus has assumed great importance both medically and bacteriologically. It has played two important roles in human infections: (1) a secondary role in pandemic influenza, acting as a secondary invader or potentiator of the virus, and (2) a primary role, producing pyogenic infections. The latter occur infrequently in adults; in children, on the other hand, *H. influenzae* is one of the more frequent causes of serious pyogenic infections. His-

TABLE 39. REQUIREMENTS OF X AND V FACTORS IN GENUS HEMOPHILUS

	REQUIRED		X AND V NEEDS NOT PROVEN
	X	V	
<i>H. influenzae</i> .....	+	+	<i>H. pertussis</i>
<i>H. Koch Weeks</i> .....	+	+	<i>H. parapertussis</i>
<i>H. parainfluenzae</i> .....	0	+	<i>Moraxella lacunata</i> ( <i>H. duplex</i> )
<i>H. hemolyticus</i> .....	+	+ or 0	<i>H. ducreyi</i>
<i>H. suis</i> .....	?	?	
<i>H. hemoglobinophilus</i> .....	+	0	

tory of the recognition of the above facts furnishes many examples of principles which are fundamental in bacteriology and exemplifies the importance of the biology of an organism for an understanding of its role in human disease.

#### ROLE OF *H. INFLUENZAE* IN PANDEMIC INFLUENZA

During the pandemic of influenza of 1890, Pfeiffer (1892, 1893) isolated from the nasopharynx of most of those suffering from the disease small straight Gram-negative bacilli tending to occur in clumps; they stained with difficulty by ordinary dyes. Loeffler's methylene blue revealed polar granules. Growth in pure culture required substances present in whole blood; the growth-stimulating substances were associated with the iron-containing portion of hemoglobin. Blood agar plates sparsely seeded yielded small transparent colonies which produced no change in the surrounding medium.

The frequency of occurrence of this organism in patients with influenza and its alleged virtual absence in normal individuals led to the erroneous conclusion that *H. influenzae* was the cause of the influenza pandemic of 1890; thus this organism was named the influenza bacillus and in 1923 was designated *H. influenzae* by The American Society of Bacteriologists. Those who have objected to this terminology have continued to use the name Pfeiffer's bacillus.

Investigations on this organism from 1892 to 1918 are reviewed by Christenson (1922) and Scott (1929). The results cast doubt upon the primary agency of the influenza bacillus in pandemic influenza; its presence was demonstrated in the nasopharynx of normal subjects and of patients with a variety of other infections of the respiratory tract during epidemic and interval periods. Two nutritional factors were shown to be essential for growth, thus distinguishing it from other known bacteria (Ghon and Preyss, 1902, 1904 and Cantani, 1901, 1902). In 1899, Slawyk cultivated an influenza bacillus from the spinal fluid of a patient with meningitis. In 1911, Cohen reported a group of patients with influenza bacillus meningitis: the organisms differed

from Pfeiffer's bacillus; the colony was more opaque; and the stained organisms were much more pleomorphic. Injection of a vaccine into rabbits produced an antiserum which agglutinated the homologous organism and protected animals against lethal infections. Wollstein (1915) extended this work and reported a larger experience with meningitis; rapid autolysis was described as a characteristic feature of strains cultivated from spinal fluid.

During the 1918 influenza pandemic extensive bacteriologic investigations were carried out to determine the role of the influenza bacillus. The results are reviewed by Jordan (1927) and Scott (1929). There is no doubt that most investigators who studied this special problem and who therefore had special interest in looking for *H. influenzae*, found a very high incidence not only in the nasopharynx but also in postmortem lung cultures. Unfortunately, the methods then available could not differentiate between the encapsulated and thus potentially pathogenic influenza bacilli and the nonencapsulated forms known to be widely distributed in the normal nasopharynx. Nor had methods then been developed for distinguishing the true influenza bacillus from closely allied species.

Study of the immunologic properties of the enormous number of strains isolated failed to separate the disease-producing strains from the others (Jordan, 1927; and Scott, 1929). The methods used were again at fault; the agglutination test, used by most students, was performed by a technic which is now known to be unsuitable for detecting type specificity; the mixtures of bacterial suspensions and diagnostic antisera were heated to temperatures above 45° C. for a period of from 2 to 6 hours. Such treatment has been shown to destroy the capsule when present and also to liberate somatic protein. Therefore, it is not surprising that great diversity of immunologic types was a common finding among all investigators. Subsequent work shows serologic cross reactions among the somatic antigens of typable and nontypable *H. influenzae*. Povitzky and Denny (1921) and Rivers and Kohn (1921), on the other hand, using the same technic but employing antisera absorbed with heterologous strains, reported 2 or 3 definite types among strains isolated from spinal fluid of patients with meningitis. Likewise, study of the immune response to the influenza bacillus of individuals with clinical influenza failed to contribute evidence on the role of this organism



because of the use of procedures unsatisfactory for that purpose.

However, certain facts were learned from the extensive bacteriologic investigations carried out during the pandemic of 1918 (Scott, 1929; and Jordan, 1927). Davis (1917, 1921), Thjotta and Avery (1921), Fildes (1921) and Rivers and Poole (1921) extended our knowledge of bacterial growth factors and standardized procedures for the use of X and V factor requirements as a diagnostic aid. In brief, they showed that whole blood contained both factors. Their action could be separated by exposing whole-blood extracts to 250° F.; V factor was thus destroyed. A yeast extract sterilized by filtration served as a good source of V factor.

A study of the nutritional requirements of strains diagnosed as influenza bacilli during the pandemic of 1918 led to the discovery of some new organisms. Pritchett and Stillman (1919) reported an organism which they labeled X bacillus; a beta type of hemolysis appeared following the growth of this organism on blood agar, and X factor was not needed for growth. Rivers (1922) described strains which he named *B. parainfluenzae*; they differed from true *H. influenzae* only in their ability to grow in the absence of X factor. Hemolytic strains requiring both X and V factors have been reported by both Fildes (1924) and Valentine and Rivers (1927). All hemolytic varieties are now classified as *H. hemolyticus*.

Search for evidence of a filtrable virus in patients with influenza also met with failure as outlined by Jordan (1927) and Scott (1929). Technics for the isolation and identification of viruses were just beginning to be explored. It is believed that the choice of patients too late in their disease to yield the virus and the use of immune individuals as recipients were responsible for the failure to transmit the virus to human beings. Nonetheless, the view was held by a number of investigators of the 1918 pandemic that its unprecedented severity reflected the concurrent interplay of a virus and the influenza bacillus. This thesis was strengthened by the recovery of a virus from swine in-

fluenza (Shope, 1931) and by the demonstration that the synergistic effect of an influenza bacillus, *H. suis*, and swine influenza virus is essential for both the natural and experimental disease. The importance of this contribution deserves emphasis: it illustrates the enhancement of injury caused by the combined effect of a bacterial and a virus infection.

It is now clear that the extensive bacteriologic investigations carried out during the 1918 pandemic of influenza failed to define the complete role of *H. influenzae*. Reports on the incidence of *H. influenzae* in the respiratory tract of patients with influenza and in lung cultures of fatal cases included not only encapsulated and non-encapsulated *H. influenzae* but *H. parainfluenzae* and perhaps even *H. suis*. The significance of the latter will be discussed shortly. All available evidence outlined by Jordan (1927) and Scott (1929) fails to present convincing support of the theory that *H. influenzae* was the primary agent. On the other hand there is a sound basis for the view that the influenza bacillus played an important role as a secondary invader. While attempts to demonstrate the presence of a virus failed, the reasons for negative results are apparent from subsequent developments. The primary agents in subsequent epidemics, differing from the great pandemic only in severity, have been proven to be viruses. Secondary bacterial invaders have not played a significant role. Whether this is due to differences in viral agents or in the prevalence of suitable bacterial agents, or in both components, cannot be answered.

The investigations of Shope (1931, 1944) raise the question whether the facts disclosed for swine influenza also hold true for human pandemic influenza. Since the swine epidemics appeared for the first time concurrently with the human influenza pandemic of 1918, Shope suggests that the latter disease originated in swine. He proved that swine influenza, both in nature and as ex-

perimentally produced, results from the combined action of 2 infectious agents; the swine influenza virus and *H. suis*. A study of the epidemiology of this disease demonstrated that the virus lies dormant in lung worms which appear to live in symbiotic relationship in the swine lungs. Under appropriate climatic conditions *H. suis* is found in increasing numbers in the nasopharynx of the experimental animals. The virus ceases to lie dormant, and epidemics of swine influenza are launched.

The clinical and pathologic similarities of swine and human influenza have led several investigators to explore the synergistic action of human influenza viruses and *H. influenzae*; the conflicting results have been reviewed and extended by Bang (1943).

The effect of *H. influenzae* on both human and swine virus deserves re-examination in light of current concepts concerning the biology of *H. influenzae*. However, it is evident from our present knowledge that the human and swine influenza viruses as well as the two varieties of *Hemophilus*, *influenzae* and *suis* possess fundamental differences. Even if it could be shown that the swine virus had been the primary cause of the 1918 human pandemic, subsequent epidemics of influenza have clearly been the results of different viruses. Evaluation of the importance of synergism between an influenza virus and bacillus in determining the severity of human pandemics must await additional study.

#### ROLE OF *H. INFLUENZAE* AS A PRIMARY PYOGENIC AGENT

The first authentic case of influenzal meningitis was reported by Slawyk (1899). The importance of this organism as a cause of meningitis was emphasized by Rivers (1922). Taylor (1927) stressed the role of the influenza bacillus in purulent arthritis. Lemierre (1936) first described the characteristic clinical syndrome which follows

when *H. influenzae* produces an obstructive infection of the lower respiratory tract.

Some fundamental differences between strains cultivated from these pyogenic infections and the majority of those isolated from the respiratory tract were first described by Cohen (1909). Wollstein (1915) described their characteristic autolysis and Scott (1929) the iridescent aspect of their colonies.

The contributions of Pittman (1931) clarified some of the controversial issues. Strains of *H. influenzae* prevalent in the healthy human respiratory tract were shown to differ from those isolated from persons with *H. influenzae* infections. It was confirmed that strains cultivated from patients with meningitis, bacteremia and pneumonia could be differentiated from nonpathogenic forms by their iridescent growth on Levinthal agar; capsules could be demonstrated by special technics. Six different types of *H. influenzae* were identified by precipitation and agglutination tests; the former method demonstrated the presence of the specific soluble substance by its precipitation with homologous diagnostic typing antiserum. The 6 types were designated *a*, *b*, *c*, *d*, *e* and *f*; type *a* specific substance was shown to be a polysaccharide. Virtually all of the meningitic strains were type *b*. Dried alcoholic precipitates of the cultures of 2 of the strains reported by Rivers (1921) were also identified as type *b*.

The agglutination reaction when incubated at 37° C. for 2 hours showed the same degree of specificity as the precipitation test. However, when agglutination was carried out at 47° C. for 4 hours (the conventional procedure), there were marked cross reactions among the different types. This fact probably explains the failure of the agglutination test, in the hands of earlier observers, to identify specific types which must have been present in a part of the population. More recent studies have shown that the somatic antigens of all 6 types as well as some nonencapsulated strains exhibit immunologic cross reactions. Exposure to the higher temperature apparently releases some somatic components. The



labile capsules of *H. influenzae* cannot be identified after such treatment. Pittman's study of variation of colonial forms of *H. influenzae* demonstrated the process whereby an S strain under artificial cultivation becomes rough; the changes observed explained earlier controversies on morphology and some of the failures to identify specific types. Fothergill and Chandler (1936) confirmed these results. It is apparent, as emphasized by Pittman, that the immunologic behavior of the influenza bacillus parallels in a number of respects that of the pneumococcus.

During the ensuing 10 years investigations dealt mainly with treatment of pyogenic infections caused by the influenza bacillus. The frequency of this organism as a cause of meningitis in children and a mortality rate of over 90 per cent had already been well established. The use of accurate methods for bacteriologic diagnosis disclosed the fact that *H. influenzae* was the most frequent cause of meningitis during years when the incidence of meningococcus infections was low. Since type *b* was found to be responsible for almost all cases of influenzal meningitis, the production and use of therapeutic antiserum naturally followed. Ward and Fothergill (1932) and Fothergill (1937) produced a therapeutic horse antiserum against type *b*. The use of this serum in 220 cases of influenzal meningitis yielded very disappointing results, despite its administration twice daily intravenously along with fresh complement and serum intrathecally; 84 per cent of the patients died. Pittman (1933) also produced a therapeutic horse antiserum against type *b*; this was used for only a small series, but it, too, was unsuccessful on the whole.

In 1939, Alexander, using methods learned in the production of pneumococcus rabbit antiserum, produced an *H. influenzae* antiserum in the rabbit. The principles shown by Dubos (1937, 1938) to be of importance in pneumococcus vaccine production were applied in an effort to produce a vaccine reflecting the chemical composition of *H. influenzae* as it occurs in human infections. Attention was focused on attaining optimal encapsulation of the organism. It was shown that encapsulated forms of *H. influenzae* could be differentiated into types by the capsular swelling phenomenon (Alexander, 1939); the technic is comparable to the Neufeld Test devised for typing pneumococci. This procedure also proved useful for detecting changes in the state of the capsule. Study of type *b* *H. in-*

*fluenzae* through all phases of the growth cycle in Levinthal broth and agar demonstrated that the capsules are much more labile than those of pneumococci. When large inocula are grown for 7 hours, the capsules begin to show deterioration, and in 24 hours it is difficult to identify them. Degeneration of the capsules takes place much more slowly when the inoculum is small, suggesting that enzymes produced by the organisms themselves are responsible. For these reasons the vaccine used to produce rabbit antibody was prepared from the luxuriant growth of *H. influenzae* resulting from 6 hours' incubation of culture on Levinthal agar. The organisms if washed from the plate with 0.5 per cent formalized saline and immediately iced exhibited good preservation of capsules for as long as a few weeks. However, degeneration of capsules occurred even in the presence of 0.5 per cent formalin if the suspensions were allowed to stand at room temperature for as short a period as 2 hours.

Heidelberger's quantitative chemical method was used for measuring antibody to *H. influenzae* in rabbit antiserum; the antibody concentration could be accurately expressed in terms of mg. of antibody nitrogen per cc. (Alexander and Heidelberger, 1940). The use of selective dosage of antibody according to the severity of the meningeal infection permitted a quantitative approach to serum therapy.

The development of a satisfactory virulence test for *H. influenzae* by Fothergill et al. (1937) provided an in-vivo method for evaluating the influence of some therapeutic agents on this organism. In our experience (Alexander and Leidy, 1943) 2 to 200 organisms of type *b* *H. influenzae* strains isolated from patients with *H. influenzae* infections, when suspended in mucin as first described by Miller (1933), are lethal for at least 50 per cent of mice infected by the intraperitoneal route. Mouse-protection tests were used to check the validity of the quantitative chemical methods for determining potency of type *b* rabbit antiserum by measuring the anticarbohydrate antibody. The protective element in the therapeutic antiserum proved to be the

anticarbohydrate (Alexander et al., 1944). The power of available agents to protect against the experimental mouse infection has also served as a good guide to their therapeutic efficacy in human disease. The results will be discussed later under Treatment.

#### MORPHOLOGY

Any description of morphology must be related to the source of the organisms. In pathologic fluids, spinal (Fig. 25), synovial or pleural, the organisms are usually predominantly coccobacillary, simulating diplococci; an erroneous diagnosis of pneumococcus is often made when Gram staining is unsatisfactory. At times the bacilli occur in short chains and are so short that they are mistaken for streptococci. Along with these forms it is virtually always possible to find definite bacilli, some quite long; at one end of some there is seen a spherical body stained only at the periphery. Occasionally, the predominant shapes are very bizarre, long slender forms occurring together with thick bacilli which assume the contour of a club, elbow or other irregular outlines. The possibility that these are variants which favor the emergence of a rough strain is suggested, but demonstration of their capsules makes such an interpretation unlikely.

In cultures the composition of the medium and the age of the culture determines to a great extent the morphology of *H. influenzae*. When Levinthal agar is seeded with 0.5 cubic centimeters of Levinthal broth culture and incubated 2 to 4 hours most of the organisms are clearly bacillus-shaped. There are also seen thick forms, irregular in outline, as if the protoplasm within were irregularly distributed, and chain formation is common. After 6 to 8 hours' incubation the short bacilli and coccobacilli predominate, and the long forms are in the minority; regularity of morphology is characteristic. Cultures which have been growing for 24 hours contain a large amount of amorphous debris, and the predominant recognizable form is the minute,

short, poorly stained coccobacillus, giving the impression that only a part of the organism takes the stain. Evidence of autolysis becomes increasingly apparent after 12 hours. At first the organisms take the stain less readily; later amorphous debris is prominent, indicating that the organisms have disintegrated. Inoculation of a Levinthal agar plate with 0.5 cubic centimeters of an 18-hour Levinthal broth culture results in growth in 3

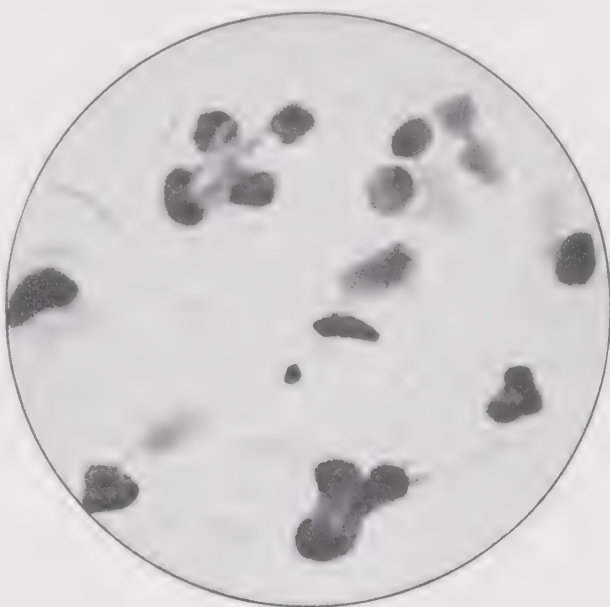


FIG. 25. Gram stain of purulent spinal fluid infected with *H. influenzae*, type b.  $\times 800$ .

to 4 hours. Iridescence is visible by obliquely transmitted light within 4 to 6 hours; this quality becomes more striking during the next 2 hours and subsequently starts to decrease. After 24 hours the iridescent quality is absent. Paralleling this phenomenon, the capsules disappear and the organisms disintegrate. There is reason to believe that these three changes which occur simultaneously are the result of liberation of enzymes by the bacteria. When a much smaller inoculum is used to seed Levinthal agar (a 2 mm. loop of 18-hour Levinthal broth culture) maximum iridescence is seen in 18 hours, the capsules are well preserved, and evidence of autolysis of organisms is absent at that time. Apparently a longer period is required for this smaller population to produce sufficient autolyzing enzymes. In Levinthal broth the changes in morphology are similar, but less pleomorphism is seen and autolysis proceeds more slowly



CULTIVATION AND BIOCHEMICAL  
CHARACTERISTICS

Growth from pathologic fluids may be obtained on blood agar or broth at pH 7.6, somewhat better on "chocolate" agar. Optimal growth takes place in media in which the contents of the red cells are liberated either by heat, as in Levinthal (1922), or by peptic digestion, as in Fildes (1920). Both of these media have the additional advantage of transparency and therefore are more suitable for the study of the characteristics of individual colonies. The presence or absence of iridescence can also be studied by viewing the growth on the surface of Levinthal or Fildes agar in obliquely transmitted light. Growth on these media is influenced by pH and availability of oxygen. The optimal pH is 7.6. Increased aeration by frequent agitation or by use of shallow layers of broth enhances growth.

The broth we have found most satisfactory is a further modification of the Pittman (1931) changes in Levinthal broth. It is made by combining one part of "Levinthal stock" with 3 parts of neopeptone broth (Lenert and Hobby, 1947). "Levinthal stock" is prepared as follows: brain heart infusion broth (Difco), made according to directions on the bottle, is heated to vigorous boiling and sterile defibrinated horse blood is added to make a final concentration of 10 per cent. The mixture is filtered through Whatman filter paper No. 12, and the clear filtrate is sterilized by Seitz filtration.

Levinthal agar is made by adding one part of sterile "Levinthal stock" to one part of melted agar [45 Gm. Proteose Agar No. 3 (Difco) plus 15 Gms. Bacto agar per liter of water].

Identification of the influenza bacillus does not depend upon the usual biochemical reactions. The bacillus lacks certain enzyme systems common to most bacterial species. Its diagnosis depends upon the need for certain growth factors X and V. Confirmation of the genus and the exact definition of type is furnished by the demonstration of its specific polysaccharide. Lwoff and

Lwoff (1937) have shown that X factor acts physiologically as hemin and that V factor can be replaced by coenzyme 1 or coenzyme 2. Subsequently Schlenk and Gingrich (1942) reported that nicotinamide nucleoside can also function as V factor.

Christensen (1922) reported that *H. influenzae* produced acid from proteins. He suggested that this fact might be responsible for the diversity of opinion concerning the ability of this organism to ferment carbohydrates. In his opinion, evidence for fermentation of carbohydrates was lacking. There is general agreement, in any event, that this function is of no differential value.

Most strains of *H. influenzae* produce indole. This is true of a larger fraction of encapsulated strains than of the nonencapsulated. There are strains in each group which show no indole production; it has proved to be too variable a characteristic to aid in the classification of these organisms.

One of the most consistent characteristics of *H. influenzae* is its ability to reduce nitrates to nitrites. Hoagland (1942) used quantitation of this action for measuring growth of *H. influenzae*.

The solubility of *H. influenzae* in bile, first described by Sellards and Sturn (1919) and confirmed by Pittman (1931) offers another point of similarity to pneumococci. This trait is characteristic of both pathogenic and non-pathogenic varieties of *H. influenzae* and therefore is of no differential value.

## ANTIGENIC STRUCTURE

The antigenic pattern of encapsulated pathogenic varieties of *H. influenzae*, types *a*, *b*, *c*, *d*, *e* and *f* has been shown to resemble that of type-specific pneumococci; a specific soluble substance is produced by each type and is concentrated in the capsule as well. Goebel (reported by Pittman, 1931) first showed that type *a* specific substance is a polysaccharide. Dingle and Fothergill (1939) first reported the polysaccharide nature of type *b* specific substance; this was confirmed by MacPherson et al. (1946) for types *a* and *b*, and shown to be true for types *c*, *d* and *f* as well. The type of a given strain of *H. influenzae* may be established

by capsular swelling, agglutination of organisms or precipitation of the specific soluble substance with diagnostic typing serum. The same antigen, the type specific polysaccharide, is responsible for these three reactions and also for stimulating the production of protective antibody as well.

It is of great interest that three types of *H. influenzae* are immunologically related to certain types of pneumococci (Chapman and Osborne, 1942; Alexander et al., 1946). Table 40 shows the cross reactions as evi-

TABLE 40. CROSS REACTIONS BETWEEN POLYSACCHARIDES OF PNEUMOCOCCI AND INFLUENZA BACILLI \*

PNEUMOCOCCUS	<i>H. influenzae</i>
6 Sub group.....	Type a
6 Sub group.....	Type b
11 †.....	Type c
15 A †.....	Type b
29 Sub group.....	Type b
35 B †.....	Type b

\* Alexander, H. E., Leidy, G., and MacPherson, C., 1946, Production of types a, b, c, d, e and f *H. influenzae* antibody for diagnostic and therapeutic purposes. *Journal of Immunology*, 54, 207.

† Not hitherto recorded.

denced by capsular swelling between the polysaccharides of *H. influenzae* and pneumococci.

The characteristics of the somatic antigens are less well known. Platt (1939) isolated two proteins: a "P" substance, making up the mass of the protein, which requires destruction of the organism for its liberation; and an "M" substance which is labile, small in amount and apparently a surface antigen since it is freed from the intact organism by washing with saline. The "M" substance is toxic for animals and is common to all strains, whereas "P" substance is nontoxic and differs among strains. Dubos (1941) obtained from an R derivative of type b *H. influenzae* which had high toxigenic power, an antigen lethal for rabbits in 0.1 mg. doses; immunization of rabbits resulted in resistance to 50 M.L.D.

of this substance. The toxic substance is believed to be an endotoxin.

MacPherson et al. (to be published) have been investigating the somatic antigens of some members of the influenza bacillus group. One of their main interests is the use of immunochemical procedures for investigating the relationship of the widespread strains of nonencapsulated *H. influenzae* to the encapsulated form of this organism and whether *H. influenzae*, *H. parainfluenzae* and *H. suis* differ significantly. The presence of a similar immunologic grouping in all types of encapsulated *H. influenzae* and in some nontypable varieties has been demonstrated by immunologic cross reactions among these somatic antigens.

#### VARIATION

When cultures of encapsulated strains of *H. influenzae* are sparsely seeded on a transparent medium (Levinthal or Fildes agar), two kinds of colonies appear, as first reported by Pittman (1931). Almost all of the colonies are opaque and large after 18 hours' growth. When viewed in obliquely transmitted light they show a characteristic iridescence. At times there are present at least one or more colonies which fail to show iridescence; they are smaller, bluish in color and transparent. Transfer of one of the latter colonies to Levinthal broth yields a culture with characteristics differing from the original; no specific soluble substance is detectable, capsular swelling cannot be demonstrated; and when the broth inoculum is again seeded on Levinthal agar no iridescent colonies are seen. These variants arise spontaneously under what is deemed optimal conditions of artificial cultivation. Under certain less favorable conditions the culture is made up predominantly of these variants and the culture is said to have passed from the smooth to the rough phase.

This change normally takes place rather slowly but can be speeded up by certain procedures described by Pittman (1931); addition of anti-S serum to the culture medium, or selection, as in the maneuver just described, of a variant colony which has appeared spontaneously. This change from S



to R can be retarded by repeated selection of iridescent colonies by daily subculture or by preserving the culture. Two methods are available for the latter purpose: drying and sealing the culture absorbed on bits of filter paper under a vacuum of 10 microns of mercury (Brown, 1926) or suspending the organisms in whole normal rabbit blood in sealed tube (Ward and Wright, 1932). Change from a rough to a smooth strain is accomplished artificially with great difficulty; it was achieved by Pittman (1931) in only one out of four strains studied, even though all methods previously found successful for converting R pneumococci to the S form were tried.

While the mechanism of these changes has not been clearly established, the evidence suggests that the rough variants arise by mutation; the procedures successful in changing cultures from smooth to rough merely select spontaneously occurring mutants. The fact that reverse mutation, involving other mutant traits, does occur makes this phenomenon seem a likely explanation of the transformation of R to S forms (see Chapter 2). This subject brings up two interesting questions: (1) Are the widespread nontypable *H. influenzae* actually variants of encapsulated varieties? (2) If *H. influenzae* plays an important role in influenza pandemics, is the conversion of rough to smooth varieties an essential part of the process? In this connection, the report by Dochez et al. (1932) is of interest. They described a change from the R form of *H. influenzae* to the S form in the nasopharynx of chimpanzees during an infection with the common cold virus. It is clear that our knowledge is inadequate for answering these questions.

In addition to the presence of these variants, which differ in certain traits from the rest of the population, changes involving the population as a whole occur during the growth cycle; some of these have already been described. In addition to the morphologic changes, there is good reason to believe that important chemical changes caused by autolytic enzymes are also taking place. There is no direct evidence that antigenicity of the specific polysaccharide is impaired, but use of suspensions of *H. influenzae* showing some morphologic changes due to autolysis results in the production

of a smaller quantity of anticarbohydrate antibody than that produced from organisms showing no autolysis; also the capsules disappear under these changes. Moreover, the proof that autolytic enzymes of pneumococci render their type-specific polysaccharides antigenically inactive suggests that the same series of events may occur in *H. influenzae* cultures.

#### TOXINS AND PATHOGENICITY

A number of authors (Jordan, 1927; and Scott, 1929) working on strains of *H. influenzae* cultivated from patients with influenza during the pandemic of 1918, reported that the injection of some strains into animals was followed by lethal toxic injury. However, the size of the lethal dose suggests that the toxic effect was due to an endotoxin and not an exotoxin.

There is no evidence that *H. influenzae* produces a true exotoxin. On the other hand, the injurious effect caused by what are probably endotoxins may play a significant role in the pathogenesis of severe infections. Whether the different pathologic potentialities described for some strains depend upon their capacity to produce this material cannot be answered. Nor do we have any evidence that antibody to these toxic substances is important in recovery from *H. influenzae* infections. On the other hand, the antigenic importance of the toxic fractions isolated by Platt (1939) and Dubos (1941) warrants further exploration.

*H. influenzae* is not naturally pathogenic for any of the smaller animals. Multiplication with invasion of the blood does occur in mice injected intraperitoneally with organisms suspended in mucin (Fothergill et al., 1937) or when suspensions in brain are introduced intracerebrally (De Torregrassa and Francis, 1941). These tests serve a useful purpose for testing efficacy of antibacterial agents but are not adequate for differentiation between pathogenic and non-pathogenic strains.

In monkeys, Blake and Cecil (1920) reported bronchiolitis and hemorrhagic bronchopneumonia following intratracheal introduction of a culture of a pathogenic influenza bacillus. Wollstein (1911) produced meningitis in monkeys by intrathecal inoculation of *H. influenzae*.

Wright and Ward (1932) described an in vitro test which could distinguish between strains cultivated from spinal fluid and those found in the respiratory tract of many normal subjects; the former were uninfluenced by diluted normal rabbit blood, the latter were killed. All strains which were well preserved in rabbit blood produced a specific soluble substance.

A number of investigators (Jordan, 1927; and Scott, 1929) reported that strains of *H. influenzae* differed in their capacity to produce death in animals from toxic injury. This question deserves further study.

On the other hand, the pathogenic potentialities of *H. influenzae* for humans is closely related to the presence of a capsule and the elaboration of a specific soluble polysaccharide; there is reason to believe that this substance exerts an influence on leukocytes not unlike that described for pneumococcus polysaccharides. Nonencapsulated *H. influenzae* seldom invades the blood at any age, whereas type *b* *H. influenzae* is one of the most frequent causes of bacteremia in infancy and childhood. The human infections caused by this organism will be described later.

#### HOST RANGE

True *H. influenzae* infections occur only in man. Closely allied forms occur naturally in animals. Gram-negative bacilli classified as *H. parainfluenzae* have been isolated from cats by Rivers and Bayne-Jones (1923). *H. hemoglobinophilus* (Friedberger, 1903) is present in large numbers in the preputial secretions of dogs. *Brucella bronchiseptica* plays the same role in dogs and some other animals as *H. influenzae* plays in man, but its physiology differs much from that of *H. influenzae*. The role of *H. suis* in swine may be quite comparable to *H. influenzae* in man, but here too the characteristics of the two organisms differ significantly. The resemblance of

function of these three apparently different organisms in three animal species raises the question of influence of host on physiology of an organism.

#### ECOLOGY

It is seldom possible to assign a primary pathogenic role to nonencapsulated *H. influenzae* in any age group. In young infants it occasionally causes meningitis or pneumonia accompanied by bacteremia; recoveries from these infections have occurred after the administration of sulfadiazine alone. Epidemic conjunctivitis has been ascribed to an organism labeled Koch-Weeks bacillus (Koch, 1887; and Weeks, 1887) which is indistinguishable from nontypable *H. influenzae*. Subacute bacterial endocarditis is at times caused by *H. influenzae* both in children and adults (Rose, 1941). The role which this organism plays in chronic lung infections and in some acute lung infections, when the blood cultures are sterile and no other bacterial pathogens are demonstrable, is a controversial question which requires more evidence to answer. According to some opinions, the influenza bacillus has a destructive action on bronchial epithelium (Zinneman, 1943).

There is a striking difference between adults and children in the pathologic potentialities of encapsulated *H. influenzae* as a primary pyogenic agent. The adult appears to possess effective resistance to it. The explanation for this difference is discussed under Immunity. However, there is reason to believe that should the normal defense mechanism be altered, as has been shown to occur in severe pandemics of influenza, a greater prevalence of severe *H. influenzae* infections in adults is to be expected.

In an attempt to learn something of the ecology of this organism, unselected patients admitted to the Babies Hospital were studied for the incidence of *H. influenzae* (Alexander, 1943a). Some form of *H. influenzae* was isolated from about 30 per cent of the children; of these strains, 19 per cent



produced iridescent growth and were therefore typable, and approximately 80 per cent of these typable strains were type *b*. Type *b* was the cause of virtually all severe *H. influenzae* infections regardless of the clinical type; a very occasional case was due to types *a* or *f*. On the other hand, it was found that type *b* causes mild infections just as frequently as severe ones. A small per cent of children without signs of infection at the time the cultures were made also harbored type *b* *H. influenzae* in the respiratory tract. History of recent infection in many of these subjects suggested that they had recovered spontaneously from an infection with this organism. Johnson and Fousek (1943) have studied the spread of these infections.

Good et al. (1943) have reported the occurrence of *H. influenzae* in members of families of children who develop meningitis. A large fraction of siblings harbor the organism; among the adults, the mother is the only member who shows their presence with any frequency. Our bacteriologic study of patients after recovery from severe type *b* *H. influenzae* infections treated by any of the available effective agents shows that this organism persists in the nasopharynx for long periods after its elimination from the spinal fluid. Silverthorne (1943) has also explored this subject.

#### CLINICAL PATTERNS

It is evident that type *b* *H. influenzae* enters by way of the respiratory tract where in most children it produces a nasopharyngitis, usually with some fever. There is reason to believe that many overcome the infection spontaneously. Others develop sinusitis or otitis media. Any portion of the lower respiratory tract may be involved, from the epiglottis and surrounding structures to the alveoli. From these foci in various parts of the respiratory tract, invasion of the blood stream occurs not infrequently. The meninges and joints are

sites of predilection for localization. Rarely pericarditis or subcutaneous abscesses result. We have recently seen a severe cellulitis involving the submental and anterior cervical regions bilaterally. The severe *H. influenzae* infections found with greatest frequency are meningitis, obstructive infections of the respiratory tract, pyarthrosis, pneumonia and empyema. These clinical patterns are described in detail elsewhere (Alexander, 1943b). While most of these infections have been reported in adults, they occur only rarely.

#### DIAGNOSIS

The importance of severe *H. influenzae* infections in infants and children may be appreciated from their frequency and potentially high mortality rates. Prior to 1938 meningitis, the commonest of these, was almost uniformly fatal: over 90 per cent died. Moreover, except in years when meningococcus meningitis occurs in epidemic proportions, *H. influenzae* is the most frequent cause of meningitis. The clinical signs of influenzal meningitis do not differ from those due to other varieties of bacterial meningitis. Therefore, etiologic diagnosis depends entirely upon a bacteriologic identification. This statement is also true for *H. influenzae* pneumonia, empyema, pyarthrosis and other less frequently occurring clinical patterns such as ethmoidal sinusitis with periorbital cellulitis and edema, pericarditis, etc.

However, the situation is quite different when *H. influenzae* type *b* is responsible for an obstructive laryngeal, laryngotracheal or laryngotracheobronchial infection. The illness presents a characteristic history, and the patient a characteristic appearance.

To date, all these patients at the Babies Hospital have been 2 years of age or older. The onset is sudden, and the course fulminating. The entire length of acute illness in those cases which end fatally is usually less than 24 hours. Mild fever and difficulty in swal-

lowing and complaint of sore throat on the part of the older children make their appearance in the course of an apparently innocuous infection of the upper respiratory tract. Dyspnea starts abruptly and increases within a few hours to such a degree as to make hospitalization and tracheotomy imperative. The characteristic picture is that of a prostrated child with dyspnea due to laryngeal obstruction. Phonation is unimpaired. The temperature is high. On examination of the pharynx there is diffuse erythema, often with evident edema, and when the tongue is pressed downward the enlarged red, misshapen, edematous epiglottis is easily seen. The obstructive syndrome described has been found in our experience only in association with type *b* *H. influenzae* infection, and therefore when observed suggests such infection immediately. Bacteremia appears to be a constant feature.

When organisms are sufficiently numerous in biologic fluids to be seen on stained smear, species and type may be identified by capsular swelling (Fig. 26) or precipitin test within a few minutes. The capsular swelling test is the simpler procedure:

A 3 mm. loopful of diagnostic typing antiserum is mixed on a cover slip with an equal quantity of pathologic fluid, enough methylene blue is added to color the drop lightly, and the preparation inverted on a hollow ground slide and sealed with oil. The cover slip may be inverted on a flat slide.

The precipitin test can also provide immediate diagnosis when organisms are numerous; when the infection is so mild that bacteria cannot be demonstrated microscopically this procedure is seldom positive. The technic is as follows:

In a small precipitin tube (50 x 6 mm.) a 1 cm. column of fluid to be tested is carefully layered by a fine capillary pipette on an equal column of diagnostic rabbit antiserum. A precipitate in the form of a white ring at the interface represents a positive test. The antiserum used and the pathologic fluid must be perfectly clear. Speed of formation of the ring varies with the concentration of specific polysaccharide in the fluid; immediate appearance of the ring denotes high concentration. This time factor may be used as an index of

severity of infection of the blood or spinal fluid.

These procedures may be used for immediate diagnosis of *H. influenzae* in spinal fluid, middle ear or joint exudate, or empyema fluid; a concentrated suspension of nasopharyngeal mucus from patients with obstructive laryngitis or pneumonia due to



FIG. 26. Typing of *H. influenzae* by capsular swelling with diagnostic typing sera.  $\times 1050$ . (Alexander, H. E., 1934, Treatment of Haemophilus influenzae infections and of meningococcus and pneumococcus meningitis. American Journal of Diseases of Children, 66, 172-187.)

this organism may reveal its presence when swelling of the capsule is demonstrated. The specimen of mucus is collected on a small cotton swab (Alexander et al., 1941) passed through the nares to the posterior pharyngeal wall where it is allowed to remain for several seconds to collect mucus; at the bedside the swab is placed in a small tube containing 0.2 cc. of sterile broth.

Identification of encapsulated *H. influenzae* in cultures from the blood or other fluid may be made after incubation, usually for 18 hours, by demonstration of capsular swelling with type specific diagnostic anti-



serum. When the latter test is negative Levinthal agar is inoculated to test for iridescence of growth. If neither capsular swelling nor iridescent quality of growth is demonstrable, diagnosis of *H. influenzae* must depend on requirement of both X and V factors for growth.

DIFFERENTIATION OF MEMBERS OF INFLUENZA-BACILLUS GROUP

**Requirements for Diagnosis.** *Gram-negative*, aerobic, non-spore-bearing, nonmotile bacilli requiring hemin, coenzyme or both for growth. The differential features necessary for diagnosing individual members of the group are outlined in Table 41.

**Examination of X and V Factor Requirements of Unknown Organisms.** For this purpose media must be available for testing the separate and combined action of X and V factors.

**X FACTOR MEDIUM.** Equal parts of "Levinthal stock" (used in Levinthal agar), autoclaved 15 minutes at 20 pounds' pressure, and melted Proteose Agar No. 3 (Difco) (45 Gm. per liter plus Bacto Agar 15 Gm. per liter). X factor is stable and can withstand this treatment; V factor is destroyed.

**V FACTOR MEDIUM.** One part of yeast extract to 9 parts of melted Proteose No. 3 Agar (Difco). Yeast extract can be prepared by the following method modified from Thjotta and

Avery (1921): Emulsify 100 Gm. of powdered brewer's yeast in 400 cc. of distilled water. Adjust pH to about 4.6, boil for 10 minutes. Filter emulsion through filter paper, adjust filtrate to pH 7.0, and filter through Seitz filter to sterilize. Transfer to a sterile container fitted with a glass stopper and seal with sterile petroleum jelly.

**COMBINED X AND V FACTORS.** One part of yeast extract to 9 parts of X factor medium.

Media are distributed in 3 cc. quantities in tubes (100 x 13 mm.) and slanted.

Cultures to be tested are grown on Levinthal agar; a loopful is suspended in 0.2 cc physiologic saline just before inoculating the separate factor media.

Pure hemin and coenzyme may also be used as X and V factors.

**Classification of Influenza Bacillus Group according to X and V Factor Requirements.**

**REQUIRE X AND V FACTORS FOR GROWTH.**

1. *H. influenzae*.

A. Typable, potentially pathogenic strains are encapsulated. They are classifiable into 6 specific types by the polysaccharide elaborated. A characteristic iridescent growth is produced on Levinthal agar.

B. Nontypable, noniridescent, nonencapsulated, seldom pathogenic organisms cannot be differentiated from encapsulated *H. influenzae* on blood agar by morphology of individual members or their colonies. Moreover, they also require X and V factors. Their failure to pro-

TABLE 41. DIFFERENTIAL CHARACTERISTICS OF INFLUENZA-BACILLUS GROUP

	CAPSULES	IRIDES- CENCE	GROWTH FACTORS		HEMOL- YSIS
			X	V	
<i>H. influenzae</i>					
Typable a-f.....	+	+	+	+	0
Nontypable.....	0	0	+	+	0
<i>H. hemolyticus</i> .....	?	?	+	+	+
<i>H. parainfluenzae</i>					
Typable.....	+	+	0	+	0
Nontypable.....	0	0	0	+	0
<i>H. hemolyticus</i> .....	?	?	0	+	+
<i>H. hemoglobinophilus</i> .....	?	?	+	0	0
<i>H. suis</i>					
Typable.....	+	+	?	?	0
Nontypable.....	0	0	?	?	0

? = Not determined.

duce iridescent growth on Levinthal agar identifies them as nontypable nonencapsulated variety.

2. *H. hemolyticus*. Production of beta hemolysis in blood agar distinguishes this organism from *H. influenzae*. Except for its rare occurrence as a cause of subacute bacterial endocarditis, no pathogenic role is recognized. It is found frequently in the normal nasopharynx.

V FACTOR BUT NOT X IS REQUIRED FOR GROWTH.

1. *H. parainfluenzae*. Human pathogenicity appears to be limited to subacute bacterial endocarditis. It is considered a normal inhabitant of the human nasopharynx. Individual organisms are morphologically similar to *H. influenzae* save for greater regularity of form and less evidence of autolysis. Colonies on blood agar are indistinguishable from those of *H. influenzae*. This class includes two groups:

A. Encapsulated *H. parainfluenzae* (Lenert and Alexander, to be published). Capsular swelling can be demonstrated with type-specific rabbit antiserum, and iridescent growth can be demonstrated on Levinthal agar. There appears to be more than one type.

B. Nonencapsulated *H. parainfluenzae*. Growth is noniridescent, and capsular swelling cannot be demonstrated.

2. *H. hemolyticus*. Production of beta hemolysis distinguishes this organism from *H. parainfluenzae*. Human pathogenicity is not unlike that described for *H. hemolyticus*, which requires both X and V factors.

*H. suis*. Essential growth needs have not been defined. This organism reacts synergistically with the virus of swine influenza in the natural and experimental disease of hogs. Human pathogenicity is unknown. Morphologically this organism does not differ significantly from *H. influenzae* and *H. parainfluenzae*. While Levinthal broth and chocolate agar provide better growth than blood agar, growth of most strains on these media is poor. Shope reported that X and V factors are essential for growth, but study of a number of strains obtained from Dr. Shope shows that some do not require X factor. In our experience the best growth has been obtained on a modified Levinthal agar with the addition of 5 per cent horse plasma or 10 per cent yeast extract (fresh).

Study of 8 strains revealed 2 different groups (Leidy and Alexander to be published):

1. Those producing iridescent growth on modified Levinthal medium. Capsular swelling occurs on exposure to homologous rabbit antibody. Use of antisera produced against 3 iridescent strains failed to reveal immunologic differences among 5 strains. No immunologic relationship was demonstrated between the type-specific antigens of *H. suis* and *H. influenzae*.

2. Those showing noniridescent growth demonstrated no type-specific characteristics. REQUIRE X FACTOR AND NOT V.

*H. hemoglobinophilus* is the only known representative of this group. Human pathogenicity has not been recognized. Friedberger (1903) first described *H. hemoglobinophilus* in chronic purulent exudate from the preputial sac in dogs.

#### IMMUNITY

The striking relationship between age and incidence of influenzal meningitis has been well established by Rivers (1922) and by Fothergill and Wright (1933). The latter authors demonstrated a close correlation between the bactericidal power of the blood of subjects in a given age group and their susceptibility to *H. influenzae* meningitis. In children aged 2 months to 3 years in whom the incidence is highest, the blood of subjects collected at random shows only a feeble bactericidal capacity towards this organism, whereas older persons have a relatively efficient lethal action and possess at the same time an appreciable immunity to infections caused by *H. influenzae* (Chart 12). Over 80 per cent of the cases of meningitis occurred in this age period. Our experience is quite comparable.

There is reason to believe that two different immune mechanisms contribute to the bactericidal effect of blood of immune subjects, complement bacteriolysis and phagocytosis. Ward and Wright (1932) reported data in support of the first, and evidence which suggested that the *H. influenzae* antibody responsible was anticarbohydrate (Wright and Ward, 1932).

Their findings focused attention on the importance of complement in the process. Dingle



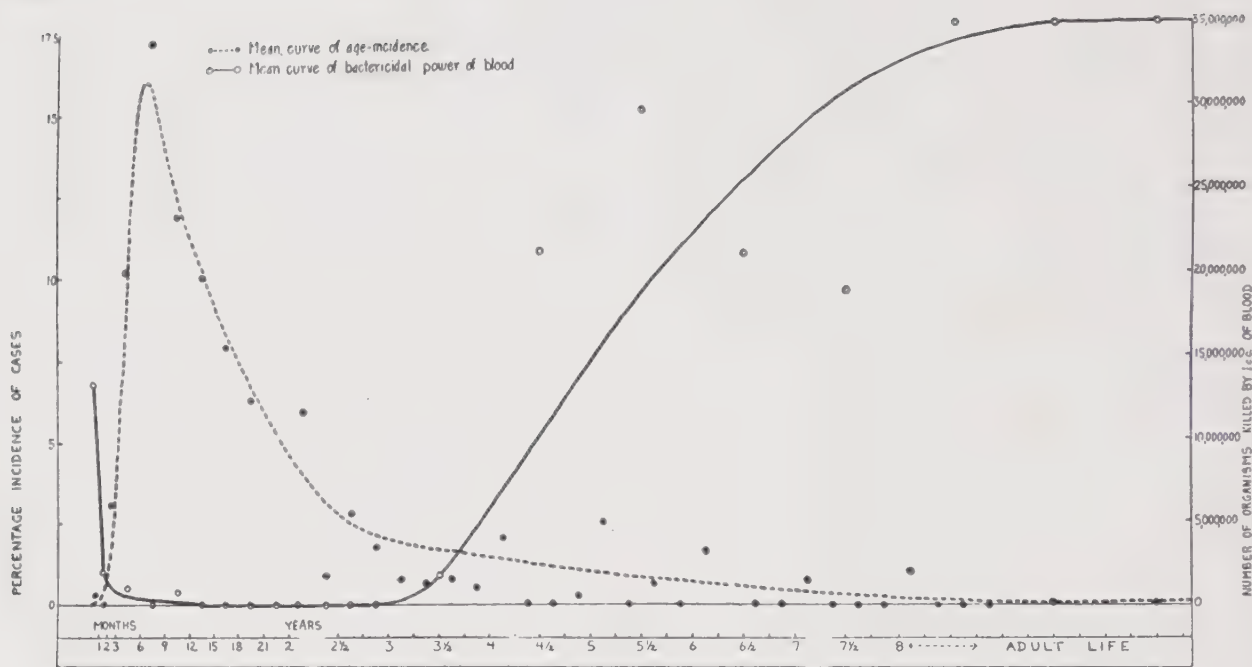


CHART 12. The relation of age incidence of influenzal meningitis to the bactericidal power of human blood at different ages against a smooth meningeal strain of *H. influenzae*. (Fothergill, L. D., and Wright, J., 1933, Influenzal meningitis. The relation of age incidence to the bactericidal power of blood against causal organism. *Journal of Immunology*, 24, 281.)

et al. (1938) confirmed the need for complement in the bactericidal action and presented evidence that the animal species from which the complement originated is of importance. Because complement was found infrequently in the spinal fluid of patients with influenzal meningitis (Fothergill, 1935), administration of fresh human serum along with horse antiserum by the intrathecal route was at one time recommended. The therapeutic results were, however, disappointing and subsequent experience by Downs (to be published) has failed to confirm the importance of complement.

Investigations on the production, measurement and use of rabbit type *b* *H. influenzae* antibody indicate that immunity to *H. influenzae* may not differ significantly from immunity to pneumococci. The protective element in type *b* *H. influenzae* rabbit antiserum is the anticarbohydrate antibody. Quantitative chemical methods originally developed for measuring anticarbohydrate antibody in pneumococcus antisera are applicable to *H. influenzae* antibody. Type *b* rabbit antiserum, administered only by the intravenous route, has

proved highly efficacious for the treatment of influenzal meningitis without the use of complement. Phagocytosis of organisms by leukocytes in the spinal fluid is apparent during treatment with this antiserum. The adequacy of dose of specific antibody for a given patient may be determined by the ability of his serum to produce capsular swelling of the organism (Alexander et al., 1942) or by his cutaneous reaction (Dingle and Seidman, 1941) to a 1/10,000 dilution of specific polysaccharide. As in the Francis test (Francis, 1933) for excess of humoral antipneumococcus antibody, the skin test with influenzal polysaccharide is positive when the circulating blood contains a demonstrable quantity of the specific type *b* anticarbohydrate.

#### TREATMENT

There are now available three effective antibacterial agents for treatment of type *b* *H. influenzae* infections: type *b* *H. influenzae* rabbit antiserum, sulfonamides, and

streptomycin. It is already evident that each one when used separately is limited in its curative effect in severe infections. On the other hand under certain circumstances each one alone can bring about recovery.

The mouse protective capacity of the agents, singly and combined, are compared in Table 42 (Alexander and Leidy, 1943 and 1947c).

TABLE 42. SUMMARY OF PROTECTIVE POWER OF THERAPEUTIC AGENTS IN MICE \*

THERAPEUTIC AGENT	PROTECTION	
	M.L.D.	NO. OF MICE
Sulfanilamide.....	500	120
Sulfadiazine.....	9,250	280
Serum.....	28,875	625
Sulfadiazine + serum.	1,000,000	270
Streptomycin.....	100,000,000	200

\* Alexander, H. E., and Leidy, G., 1947, The present status of treatment for influenzal meningitis. American Journal of Medicine, 2, 457.

Sulfadiazine, the most effective of the sulfonamides tested, is limited in its capacity to overcome the infection when the inoculum exceeds 10,000 M.L.D. The efficacy of sulfathiazole, sulfamerazine and sulfapyridine can be expected to be comparable with sulfadiazine. Specific rabbit antiserum alone protected against an average of about 30,000 M.L.D. When both agents were used in combination the mice could regularly withstand 1,000,000 M.L.D. Streptomycin alone, on the other hand, could eliminate 100 million M.L.D. In vitro the influence of streptomycin on *H. influenzae* is equally impressive; a rapid lethal action takes place (Alexander and Leidy, 1946, and also to be published). There is a striking correlation between the in-vitro and in-vivo susceptibility of this organism.

The selection and combination of these effective agents for treatment of a given patient depend to a great extent on the lo-

cation and severity of the infection. For example, in certain clinical patterns—e.g., pneumonia in infants older than one year, most obstructive infections of the lower respiratory tract, early purulent otitis media and ethmoidal sinusitis with periorbital cellulitis and edema—sulfadiazine is promptly successful even though bacteremia is almost invariably present. In meningitis and pyarthrosis, on the other hand, there are clear indications for using the combined action of at least two of these three agents.

Nevertheless, it is clear that a certain proportion of patients with meningitis do recover on sulfonamides alone. Our own clinical experience suggests that this fraction is small. The evidence suggests that the capacity of the sulfonamide compounds to effect complete recovery is conditioned on two factors: the use of the drug must be started early in the course of the infection, and the infection must be of relatively mild degree. A minimum of two weeks of such treatment is essential for elimination of infection. It is of interest that during a period when approximately 30 patients were treated, only 2 fulfilled the criteria which justified the use of sulfonamides alone. Reports of visiting physicians from Europe indicate that the present mortality rate there in influenzal meningitis is 75 per cent; sulfonamides have been the only available therapeutic agents.

The use of combined therapy with specific rabbit antiserum and sulfadiazine over a 10-year period has proved highly successful. The application of certain principles has not only increased its efficacy but has greatly simplified its use. The dose of antibody needed varies with the severity of infection. Therefore, some objective criterion of severity is essential. Moreover the sufficiency of the original dose decided upon requires confirmation.

1. The best index of severity of infection is the concentration of sugar in the spinal fluid withdrawn before treatment; the lower the concentration, the greater the severity.



2. The antibody in the therapeutic rabbit antiserum is measured by the quantitative chemical method for determining mg. of agglutinin nitrogen per cc. Thus, it is possible to formulate a quantitative approach as shown in Table 43.

TABLE 43. SCHEDULE OF DOSAGE BASED ON SPINAL-FLUID SUGAR \*

SPINAL FLUID SUGAR (MGM. PER CENT)	MGM. ANTIBODY NITROGEN INDICATED
<15	100
15-25	75
25-40	50
Over 40	25

\* Alexander, H. E., Ellis, C., and Leidy, G., 1942, Treatment of type-specific *Hemophilus influenzae* infections in infancy and childhood. *Journal of Pediatrics*, 20, 673.

3. This plan aims at introducing at one time the amount of antibody necessary for recovery. Nevertheless, it is necessary to check its sufficiency. The capsular swelling capacity of the patient's serum following treatment is a good guide. This test is performed daily through the period of activity of infection; and unless it can be shown that the patient's serum contains an excess of free antibody sufficient to cause capsular swelling of the organisms when diluted 1:10, another dose of antiserum is administered (25 to 50 mg. of antibody nitrogen).

The therapeutic program just described is greatly simplified by the fact that prompt recovery follows introduction of the antiserum by the intravenous route only. When patients are treated early with the combined therapy of sulfadiazine and type-specific rabbit antibody according to the principles outlined (Alexander, 1944), the response has been so consistent that it is possible to predict not only the outcome but the course of recovery. Even in the fulminating cases in which the meningitis progresses so rapidly that the spinal fluid

sugar falls to less than 15 mg. per cent within 24 hours of onset, prompt recovery can be expected in all instances if sufficient antibody is administered in the initial dose. Actually only 80 per cent of the 90 patients treated according to this regimen recovered, the failures being attributable to delay in diagnosis and to unwarranted confidence in the value of sulfonamides alone prior to the application of the combined therapeutic program.

With regard to streptomycin a standardized program has been tested, in which patients are given 40 mg. of the drug per Kg. of body weight per day intramuscularly, divided into 8 doses and a daily intrathecal injection of 25 mg. (Alexander et al., 1946). The results in the treatment of influenzal meningitis justify certain conclusions.

In those patients whose original spinal fluid sugar concentrations are significantly above 15 mg. per cent one can expect prompt and complete recovery following the use of streptomycin alone. In severe meningitis, on the other hand, as evidenced by a concentration of spinal fluid sugar below 15 mg. per cent, streptomycin alone usually fails to bring about recovery; one of the most frequent causes of failure is emergence of resistance of the organism to the drug (Alexander and Leidy, 1947a).

Investigation of the origin of the resistant members responsible for therapeutic failure of streptomycin indicates that all strains of *H. influenzae* before exposure to streptomycin contain a minute fraction of cells which can thrive in concentrations of over 1,000 mcg. per cubic centimeter of the antibiotic. These resistant variants apparently present in large populations of all sensitive strains of *H. influenzae* have been proven to originate by mutation (Alexander and Leidy, 1947b).

Therefore, we can expect the continuous random occurrence of resistant mutants in patients if the disease is sufficiently severe or, in other words, if the bacterial population is large enough. The rate of occurrence of the resistant mutants did not differ significantly among 10 strains studied. Therefore, the emergence of resistant strains in 3 patients in

whom streptomycin failed to effect a cure cannot be satisfactorily explained by a greater frequency of occurrence of mutations. The effect of unsuccessful streptomycin treatment is to permit the selective survival and multiplication of resistant strains, the susceptible strains having been killed off.

The resistant trait is transmitted unchanged in degree through many generations. Therefore, the appearance of a few mutants in a patient during treatment can lead to an intractable infection which is uninfluenced by streptomycin. Moreover, the persistence of resistant organisms in the nasopharynx of patients whose strains emerged resistant during streptomycin treatment constitutes a significant public health problem. In one streptomycin-treated patient, followed at intervals for one year after recovery from meningitis, all of the cultures of *H. influenzae* isolated from his nasopharynx showed resistance to 1,000 mcg. of streptomycin per cc.

It is of great significance therapeutically that the mutants resistant to streptomycin are in general sensitive to sulfadiazine. The efficacy of the combined action of streptomycin and sulfadiazine is now well established. One limitation of this therapy must be emphasized: when sulfadiazine-resistant strains are present this treatment cannot be expected to be successful.

In summary, there are now available two therapeutic programs which have proved to be highly successful even in severe infections: the combined action of type-specific rabbit antiserum and sulfadiazine, and the use of streptomycin in conjunction with sulfadiazine. In a group of 90 patients treated by the former, the recovery rate was 80 per cent; in 45 patients receiving streptomycin—either alone, along with sulfadiazine, or in combination with serum and sulfadiazine—the recovery rate was 86 per cent. The choice between these two forms of treatment must weigh the expense of the serum against the toxicity of streptomycin

(McDermott, 1947; Farrington et al., 1947; and Fowler and Seligman, 1947). Even when the duration of treatment is reduced to 4 days a significant number of children show evidence of damage to the vestibular apparatus. Since the action of streptomycin on *H. influenzae* in vitro is bactericidal, success of a shorter period of treatment is anticipated. Unless it can be shown that the infection can be eliminated by a shorter period of treatment or a smaller dose which will not produce vestibular damage, the use of streptomycin must be limited to those patients not responding to the combined action of serum and sulfadiazine.

### H. DUCREYI

This organism was first described by Ducrey (1890) in the purulent discharge from the venereal disease "soft chancre" or "chancroid." Proof of the agency of *H. ducreyi* in this infection was furnished by the investigations of Greenblatt (1938). Recognition of this organism as the etiologic agent is important since the disease occurs frequently and may be confused with syphilitic chancre. Moreover, prompt recovery follows the use of sulfonamides.

The morphology of the organisms in local lesions is characteristic; chains of small Gram-negative bacilli occur in strands. Cultivation proved to be so difficult that very few pure cultures were obtained until Teague and Deibert (1920) succeeded in growing the organism in pure culture from 140 of 274 sores. All details of the simple method described must be applied for best results. Greenblatt (1938) recommends cultivation in whole defibrinated rabbit blood, incubated under partially reduced oxygen tension.

A saline suspension of killed *H. ducreyi* has served as a good antigen for diagnostic cutaneous tests. Greenwald (1943) reviews the value of diagnostic procedures and therapy.



## MORAXELLA LACUNATA

This organism was described independently by Morax (1896) and Axenfeld (1897) as a Gram-negative bacillus, occurring characteristically as a diplobacillus in the pus from conjunctival and corneal infections in man. It has been designated by various names; Morax-Axenfeld bacillus is the most familiar one. The correct name is now *Moraxella lacunata* (Bergey, 1948). Its importance as a cause of conjunctival infection is reported by Thygeson and Braley (1943). The fact that cases of *M. lacunata* and *H. influenzae* (Koch-Weeks Bacillus) conjunctivitis occur most frequently in areas where virus eye infections are widespread

raises the question whether the bacteria are the primary agents.

Isolation of *M. lacunata* is difficult initially. Loeffler's coagulated blood-serum medium is best for this purpose; colonies appear after 24 to 36 hours and are surrounded by concentric indentations caused by liquefaction of the medium, one of its distinguishing characteristics. Later growth may be obtained on blood and chocolate agar. The growth requirements have not been defined, but the organism is clearly a strict parasite. Oag (1942) has described both hemolytic and nonhemolytic varieties and studied their antigenic structure. Their biochemical reactions and pathogenicity are recorded.

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## 24

# The Pertussis Group

### HEMOPHILUS PERTUSSIS

Whooping cough bacilli (*Hemophilus pertussis*) are Gram-negative organisms generally placed in the genus *Hemophilus* (family of *Parvobacteriaceae*) of which *Hemophilus influenzae* is the type species. Primary isolation requires growth factors contained in blood, but the avirulent rough variant grows readily on ordinary media. *Hemophilus pertussis* is the causative agent of whooping cough. This highly communicable disease is an acute infection of the respiratory tract characterized in its typical form by a series of repeated spasmodic coughs followed by a sudden forceful inspiration (the whoop) and sometimes by vomiting. It is prevalent among infants and children and causes high mortality among infants and serious pulmonary and cerebral complications.

### HISTORY

It is probable that whooping cough was recognized during the Middle Ages for Moulton mentioned the treatment of a condition known at that time as the "kink," a Scottish colloquialism synonymous with fit or paroxysm. It was also known as "chin-cough" which seems to have been derived from the teutonic work "Kindhoest" meaning "child's cough." De Baillou (1578) is credited with the first classic description of the disease:

The lung is so irritated so that every attempt to expel that which is causing trouble, it neither admits the air nor again easily

expels it. The patient is seen to swell up, and as if strangled hold his breath tightly in the middle of his throat. . . . For they are without this troublesome coughing for the space of four or five hours at a time, then this paroxysm of coughing returns, now so severe that blood is expelled with force through the nose and through the mouth. Most frequently an upset of the stomach follows.

One of the earliest American descriptions was that by Benjamin Waterhouse (1822) entitled *An Essay Concerning Tussis Convulsiva or Whooping Cough*. A most comprehensive account of the disease may be found in Lapin's Whooping Cough (1943).

The causative organism, *H. pertussis*, was first observed and cultivated by Bordet and Gengou (1906) as a small Gram-negative ovoid bacillus abundantly present in the sputum of an infant with whooping cough. Rhea (1915) and Smith (1913-1914) pointed out that lesions similar to those reproduced by Mallory and Horner in 1913 may have been caused by *Brucella bronchiseptica*. Chievitz and Meyer (1916) introduced the cough-plate method for bacteriologic diagnosis. Renewal of interest in the organism occurred in 1931 when Leslie and Gardner stressed the differences in the antigenic phases during artificial cultivation.

The specificity of the etiologic agent was questioned by the suggestion that perhaps a virus shared at least a dual etiologic relationship (McCordock, 1932, 1937; Rich, 1932). It appears now certain that *H. per-*



*tussis* alone can produce the disease either in laboratory animals (Shibley, 1934) or in man (MacDonald and MacDonald, 1933).

### MORPHOLOGY

*H. pertussis*, when first isolated from an active case of whooping cough, is a small nonmotile, ovoid bacillus. The mean length is 0.5  $\mu$ , which is also the mean length of *B. parapertussis* and of *Brucella bronchiseptica* (Eldering and Kendrick, 1938). The cells are more uniform in size than those of *H. influenzae* but become pleomorphic, longer, and thread-shaped in subcultures. In liquid media a ropy and mucoid mass results from the growth of old cultures. Electron micrographs of the bacteria reveal a central mass of dense material surrounded by a wide outer area of less dense material which is said to contain the specific antigenic substance. The organism possesses a capsule.

*H. pertussis* is Gram negative, staining best when the counter stain is left on for two minutes. Toluidin blue reveals bipolar metachromatic granules, said to represent unequal distribution of cell lipids. Sometimes a granule is seen in the center of the cell.

The Toluidin blue stain can be prepared as follows:

Dissolve 5 grams of Toluidin blue in 100 cc. of absolute alcohol and 500 cc. of distilled water. After the dye is dissolved, add 5 grams of phenol and shake. Filter after 2 days. Fix film by flaming. Stain 2 minutes, wash in distilled water and dry. Freshly isolated ovoid cells serve best for demonstrating bipolar bodies.

For the purpose of demonstrating capsules, Lawson's (1940) modification of Smith's stain may be used as follows:

A loopful of growth taken from the surface of Bordet-Gengou medium is smeared in a thin film, air dried, and covered with a 5 per cent aqueous solution of phosphomolybdic acid. After 30 seconds, the preparation is

washed off in running water and then it is washed with methyl alcohol. The slide is then covered with 15 drops of a staining solution composed of 2 parts Wright's stain and 1 part glycerin, freshly made up and mixed. After contact for 2 minutes, 25 drops of distilled water are added and mixed with the stain by using a capillary pipette. The film is stained for 15 minutes, rinsed, dried and examined. In thin areas of the preparation, the capsule appears as light sky blue, while the organism is magenta or red in color.

### CULTIVATION AND FERMENTATION REACTIONS

Primary isolation can be obtained on complex media, such as that used originally by Bordet and Gengou, consisting of a potato-blood-agar-glycerol mixture or some modification of it. The optimal temperature for growth is from 35° to 37° C.

A solid medium which has proved satisfactory is prepared as follows:

1. Bordet-Gengou Agar Base, dehydrated Bacto. (Prepared by the Difco Laboratories Inc., Detroit.) This base contains the following ingredients per liter:

Potato, infusion from . . . . .	125 Gm.
NaCl . . . . .	5.5 Gm.
Proteose-peptone, Difco . . . . .	10.0 Gm.
Bacto-agar . . . . .	20.0 Gm.

2. Solution of 1 per cent glycerol in distilled water.

3. Freshly withdrawn (not over 6 hours) defibrinated sheep's blood.

(a) Suspend 4 Gm. of the dehydrated agar base in 100 cc. of 1 per cent solution of glycerol in distilled water. Heat to boiling to dissolve the medium completely. Sterilize in autoclave at 15 lbs. pressure (121° C.) for 20 minutes. This base may be stored in 100 cc. Erlenmeyer flasks in the icebox.

(b) To prepare the final medium, heat the base prepared under (a) in a water bath until completely liquefied. Cool to from 45° to 50° by placing the flask in a water bath and add the blood to make a concentration of from 20 to 25 per cent and pour plates. Use plates for cultures which have been prepared within 72 hours.

A satisfactory fluid medium has been described by Cohen and Wheeler (1946).

Casamino acids.....	10 Gm.
NaCl.....	2.5 Gm.
Monopotassium phosphate.....	0.5 Gm.
MgCl <sub>2</sub> .....	0.4 Gm.
Starch soluble.....	1.5 Gm.
CaCl <sub>2</sub> (1% sol.).....	1.0 ml.
Ferrous sulfate (0.5% sol.).....	2.0 ml.
CaSO <sub>4</sub> (0.05% sol.).....	1.0 ml.
Cysteine hydrochloride (1% sol.)....	2.5 ml.
Yeast dialysate.....	50.0 ml.
Distilled H <sub>2</sub> O to make.....	1 kg.

To prepare the yeast dialysate make a paste by mixing 500 grams of brewer's yeast (Fleischmann pure, dry type 2019) with 800 cc. of distilled water and transfer to a cellophane tube (550 mm. long, 115 mm. in diameter, and 0.089 mm. in wall thickness). Tie each end of the tube with heavy twine, immerse it in 2,000 cc. of distilled water in such a manner that the liquid cannot spill or seep through the ends of the tube. Adjust its position to give approximate equality of liquid level inside and outside the tube. Heat at a temperature of from 78° to 80° C. for 7 hours. Remove the tube and transfer the dialysate to a bottle. Add 5 cc. of chloroform per liter and store in cold room. The dialysate is clear, requires no filtration and remains satisfactory for use after several months' storage.

Dissolve the casamino acids, salt, phosphate, and MgCl<sub>2</sub> in part of the water. Add remaining ingredients and make up to 1 Kg. Adjust pH to 7.2 or 7.3. Boil until starch is dissolved and medium is clear. Filter and autoclave at 10 lbs. for 15 minutes. Inoculate medium with heavy seeding and incubate at 37° C. for 48 to 72 hours.

The organism does not produce gas but produces some acidity in glucose and lactose. It has no action on galactose, maltose, levulose, sucrose or inulin. Indole is not formed, and nitrates are not reduced to nitrites. It produces catalase but less than *B. paraptussis*. When grown in litmus milk it produces alkalinity after 10 to 14 days as compared with 1 to 4 days in the case of *B. paraptussis* or *Brucella bronchiseptica*.

#### SEROLOGIC REACTIONS AND ANTIGENIC STRUCTURE

Strains of *H. pertussis* on primary isolation constitute a single antigenic type

(Coffey, 1934; Shibley and Hoelscher, 1934). However, on artificial media variant forms occur, which Leslie and Gardner (1931) designated as, Phase 1, the virulent S-form; Phase 4, the completely avirulent form; and Phases 2 and 3, intermediate serologic variants.

The organism can be identified serologically by the agglutination test using antisera prepared by immunizing rabbits with strains representing the various phases. In this connection it has been observed that a Phase-3 antiserum will not react with Phase-1 organisms; but a Phase-1 antiserum will agglutinate Phase-3 organisms. Phase-2 organisms are rarely encountered. Organisms belonging to Phase 1 differ markedly from those of Phase 4 in morphology, colony type, hemolytic activity and antigenicity. The practical significance of this serologic classification concerns the importance of using only Phase-1 organisms for the preparation of vaccines to be used for active immunization.

At the present time the three antigenic components recognized are the agglutinin, a heat-labile toxin and a heat-stable toxin.

The surface of the cell or its capsule yields most of the agglutinin which can be prepared by extracting *H. pertussis* cells at 56° C. at pH 1.8 (Smolens and Mudd, 1943). When prepared by this technic, it possesses the following characteristics: it is antigenic, producing specific agglutinins against *H. pertussis* when injected intravenously into rabbits; it quantitatively absorbs agglutinins from *H. pertussis* antiserum; it is nontoxic; it may be used as a skin test for susceptibility; and it stimulates active immunity to pertussis. Agglutinin may also be extracted by sonic disruption of the cells (Flosdorf and Kimball, 1940).

The heat-labile toxin (Flosdorf et al., 1941) is completely destroyed at 56° C. for 30 minutes. It possesses strong necrotizing properties when injected intradermally and is lethal to rabbits when injected intrave-



nously in a dosage of 1 mgm. Disruption of the bacterial cell gives maximal yields of heat-labile toxin, but an appreciable amount is contained in the surface washings of the freshly isolated organisms (Katsampes et al., 1942). A relatively pure toxin prepared by Streat et al. (1941) has been used as skin-testing material for susceptibility.

The heat-stable toxin is probably of intracellular origin and is only partially destroyed by heating at 100° C. for 60 minutes. Little is known of its nature and properties.

The nonvirulent variant phases of the organisms contain the same toxins as Phase 1 but in only one-tenth the amount (Flossdorf and McGuinness, 1942).

#### HOST RANGE AND PATHOGENESIS

*H. pertussis* is an obligate parasite, capable of surviving for only a short time outside the human host. It is sensitive to desiccation, although viable organisms have been demonstrated in dried sputum after several hours. It is killed in 30 minutes at from 50° to 55° C. and very quickly by ultra-violet light and by chemical antiseptics. The mode of transmission is by droplet infection. No intermediate vector has been recognized. Although the human carrier is said to play a minor role the universal dissemination of the disease suggests that the carrier state may be more important than generally realized.

The organism rapidly multiplies upon the epithelial surface of the respiratory tract and invades the alveoli and their cellular walls, but seldom invades the blood stream or middle ear. The latter, however, often becomes secondarily infected with other organisms. Virulent strains can infect a variety of experimental animals: chimpanzee (Rich, 1932; Shibley, 1934), monkey (Inaba, 1912; Sauer and Hambrecht, 1929; North et al., 1940), puppy (Inaba and Inamori, 1934), rabbit (Sprunt, Martin and McDearman, 1935-1938), rat (Hornibrook and Ashburn,

1939), mouse (Burnett and Timmins, 1937; Bradford, 1938; Lawson, 1939), ferret (Cullata, 1938), and chick embryo (Gallavan and Goodpasture, 1937; Shaffer and Shaffer 1946). In the mouse and chick embryo secondary invasion of the blood stream is frequent, and infection may result from inoculation with few organisms. The clinical course of pertussis is variable but consists of three indistinct stages; the catarrhal, the spasmodic, and the convalescent, each lasting for approximately two weeks.

In the catarrhal period, which begins about 13 to 15 days after exposure, the cough is mild but progressive. The local reaction caused by the organism and its toxic components in the epithelium of the trachea and bronchi results in the early catarrhal symptoms such as coughing and sneezing. The essential lesion according to Gallavan and Goodpasture is necrosis of the midzonal and basilar portions of the epithelium with infiltration by polymorphonuclear leukocytes. As the disease progresses organisms may be found in the deeper portions of the bronchial tree, the bronchioli and the alveoli. Peribronchiolitis and interstitial pneumonia, which are common findings, now are recognized as possible results of direct infection with *H. pertussis*. Intra alveolar exudate and localized suppurative lesions are often caused by secondary invaders such as staphylococci, pneumococci, influenza bacilli, and streptococci. Edema and hemorrhage are often present in the lung parenchyma. Obstruction of the lower air passages by mucous plugs induces atelectasis, which, along with interstitial pneumonia, prevents proper oxygenation of the blood. The resulting anoxemia is probably an important factor in the causation of convulsions, occurring in about 8 per cent of hospitalized cases. The exact mechanism responsible for postpertussis encephalitis is not known.

It is probable that the heat-labile toxin is responsible for the characteristic hyperlymphocytosis, the early necrosis of bron-

chial epithelium, the early changes observed in the hilar pulmonary areas, and certain hemorrhagic manifestations.

The possible role of allergy in this disease has been defended by Toomey (1938) who claims that the organism in its Phase 1 causes the early catarrhal stage of the infection but becomes less virulent during the paroxysmal stage when the cells of the host become sensitized. This would result in the production of the ropy, tenacious, mucoid exudate which characterizes this period of the disease. The chief objection to this theory is that organisms isolated late in the disease do not appear to differ from those isolated in the early stage.

#### IMMUNITY

One attack of the disease usually results in permanent immunity. Bacteriologically proved instances of second attacks are rare, but more general application of improved culture methods may be expected to reveal their more frequent occurrence, particularly among adults intimately exposed to active cases. There is little or no resistance to infection transmitted from the mother to her infant, although the latter occasionally possesses transplacentally transferred humoral antibodies. Indeed, early infancy is a period of great susceptibility and of highest mortality.

The relative importance of humoral and cellular immunity is unknown. Antibodies as measured by complement fixation, agglutination, opsonocytophagic, and mouse-protective tests, appear in the blood during convalescence from an attack and as a result of active immunization. They remain demonstrable for a period of several months. While infection seldom occurs in the presence of a significant titer of humoral antibodies, immunity is known to exist in individuals with little or no demonstrable antibodies. The blood of both immune and nonimmune individuals possesses a consid-

erable bactericidal power (Fothergill and Walker, 1939). It is probable that immunity may depend, at least in part, on a cellular mechanism, perhaps concerned with local tissue resistance in the respiratory tract. The relative importance of antibacterial and antitoxin immunity is also undetermined, but it is probable that both play significant roles.

At the present time no entirely satisfactory method of testing susceptibility to the disease is available. In-vitro antibody tests are not sufficiently sharp, and the skin tests with heat-labile toxin or agglutino-gen require further evaluation.

The recent demonstration by Keogh, North and Warburton (1947) that freshly isolated strains of *H. pertussis* possess hemagglutinins for the erythrocytes of man, mouse and other species; and that resistance to infection parallels the development of antihemagglutinins may offer a more accurate method for the measurement of humoral immunity.

#### DIAGNOSIS

Bacteriologic diagnosis can be made by the cough-plate method or by the nasal swab method (Fig. 27). With the cough-plate method the culture is obtained by holding the plate containing the medium about six inches from the mouth of the patient during a paroxysm. With the nasal-swab method, the swab is passed through the nasal aperture until it touches the posterior pharyngeal wall. The charged swab is then passed several times through a drop of penicillin solution previously placed upon the surface of Bordet medium. The material is further streaked over the surface of the medium with a fine flexible platinum loop. After incubation at 36° C. for 3 days, characteristic colonies of *H. pertussis* appear particularly in the zone where the growth of contaminating organisms is inhibited by the penicillin. The organism is



identified by staining and by agglutinative reaction with specific antiserum.

Since humoral antibodies do not appear until the third week of the disease their demonstration is seldom of practical diagnostic value.

Numerous attempts to develop a satisfactory diagnostic skin test have been made.

Clinical diagnosis is facilitated by a history of exposure; it is based on the following signs: a progressive cough, often nocturnal at first, but later diurnal; spasmodic coughing spells followed by a forced inspiration, the whoop; and sometimes vomiting. The total white-blood-cell count reaches 15,000 to 30,000 per cc. with a relative and



FIG. 27. Nasal culture method, showing the inhibiting effect of penicillin on the growth of contaminating organisms (*right*) to facilitate the identification of *Hemophilus pertussis* colonies on the surface of Bordet's medium. The control plate is shown on the *left*. (Bradford, W. L., Day, E., and Berry, G. P., 1946, Improvement of the nasopharyngeal swab method of diagnosis in pertussis by use of penicillin. *American Journal of Public Health*, 36, 468.)

Two antigenic components of the organism, the heat-labile toxin and the agglutinin have been recently studied. Sauer (1946) states that a positive test to the agglutinin correlates with a satisfactory complement-fixing antibody titer. Furthermore, this test has been found by clinical trial to be a satisfactory method of detecting susceptibility to the disease (Felton et al., 1946). A positive reaction to the agglutinin indicates immunity, while a positive reaction to the toxin is said to indicate susceptibility. However, final assessment of the reliability of these tests requires additional study.

absolute preponderance of lymphocytes. This change in the blood count appears near the end of the catarrhal period of the disease.

Other conditions which produce coughs resembling those of pertussis are: sinusitis, infected adenoids and tonsils, allergic bronchitis, influenza, bronchopneumonia, atypical virus pneumonia and mediastinal lesions, such as enlargement of the peribronchial lymph nodes. Parapertussis (Eldering and Kendrick, 1937; Bradford and Slavin, 1937) is an acute infection of the respiratory tract which usually resembles mild pertussis and can only be differentiated by bacteriologic

methods. Instances of infection with *Bruccella bronchiseptica*, *H. influenzae* and *Bruccella abortus* have been mistaken for pertussis.

#### TREATMENT

Hyperimmune human serum (McGuinness, 1944), injected intramuscularly in doses of from 20 to 40 cc., and immune rabbit serum (Bradford, 1941) administered similarly (after preliminary testing for sensitivity) in doses of 5 to 10 cc., confer a humoral antibody level which compares favorably with the titer usually observed in convalescence. These antisera have given good clinical results. A gamma globulin prepared from hyperimmune human serum also appears therapeutically effective but requires further clinical testing.

Sulfadiazine in doses of 0.1 Gm. per kilogram of body weight per day is protective. It is frequently given in combination with immune serum. Obviously it is also effective against such common secondary invading organisms as pneumococci, influenza bacilli, streptococci and staphylococci. *H. pertussis* is susceptible to streptomycin (Bradford and Day, 1946) which protects mice against the experimental disease. Streptomycin administered to infected infants in the form of nasal drops and as an aerosol brings about a rapid disappearance of the organism from the respiratory tract (Bradford et al., 1947). Penicillin may be used effectively when susceptible organisms are present as secondary invaders.

There is little evidence that the injection of an antigen is therapeutically effective except in individuals who have previously received partial protection by active immunization. In this case, an additional injection during the catarrhal period may cause an immediate rise of the humoral antibody titer.

#### EPIDEMIOLOGY

Whooping cough exists sporadically and endemically in most of the thickly popu-

lated areas of the world. It has a tendency to occur epidemically at intervals of from 2 to 4 years. It occurs in the United States at all seasons of the year, with a peak in the northern states during the early winter months and in the southern states during the spring months. In certain localities it tends to follow the incidence curve of the common diarrheal diseases. The communicability rate in family exposure is about 85 to 90 per cent; in school room outbreaks it is from about 25 to 50 per cent. This approaches the communicability observed in certain virus diseases such as measles and chickenpox.

Although the recorded morbidity rate in the United States has increased from 1910 to 1939 (probably as a result of better case reporting), the mortality rate has definitely decreased during the past four decades. The incidence varies in different regions of the country. Morbidity as well as mortality is higher among females.

Pertussis may occur at any age, including the newborn period and old-age group. About 50 per cent of all cases are observed under 4 years of age; about 10 per cent among infants under one year. Nearly all deaths from whooping cough occur during infancy and childhood. According to Dauer (1943), the percentage of deaths by age in the United States during the period from 1935 to 1939 was as follows:

AGE	PER CENT OF DEATHS
Under 5 months.....	40.0
6 to 11 months.....	24.2
1 year.....	21.8
2 years.....	7.1
3 years.....	3.2
4 years.....	1.7
5 to 9 years.....	2.9
10 to 14 years...	0.5

In New York State pertussis accounts for about 5 per cent of the infant mortality. The general fatality rate for the United States is about 2.3 per cent. The mortality rate is higher in rural than in urban com-



munities and is increased by poor housing conditions and inadequate professional care.

### CONTROL MEASURES

The effective control of whooping cough requires early diagnosis, proper isolation of the active case, and of the exposed susceptible individual, and an adequate program for active immunization.

Since the onset of the disease is often insidious and its course variable, diagnosis is delayed longer than in most other common communicable diseases. Early diagnosis is important because the patient is most infective in the early stage of the disease. The need for a practical test for susceptibility, such as the recently developed skin test appears to be, is obvious. Physicians should apply methods of bacteriologic diagnosis, and health units should make culture facilities available for this purpose.

The patient should be isolated for a period of 6 weeks from the beginning of the disease. Ideally, a negative culture should precede release. Following exposure, susceptible children should be isolated for a period of 2 weeks corresponding to the incubation period of the disease. Exposed, susceptible infants should be passively immunized by intramuscular injection of 20 to 40 cc. of hyperimmune human serum or 5 to 10 cc. of immune rabbit serum. The exposed child previously vaccinated should receive a booster dose in the form of a single subcutaneous injection of vaccine.

An adequate program of active immunization involves the routine vaccination of all infants. This is usually carried out at the sixth month of life, but recent data suggest that the procedure may be effectively instituted as early as the first month. The total dose of vaccine should be 60 to 80 billion organisms distributed in 3 injections at monthly intervals. A booster single injection of vaccine given a year after the original course of injections is desirable.

The vaccine may be injected along with diphtheria and tetanus toxoids.

Serious complications are rare when the disease occurs in a previously immunized child, as sometimes happens.

### HEMOPHILUS PARAPERTUSSIS

*Hemophilus parapertussis* is a short, ovoid, Gram-negative, nonmotile bacillus which, in many respects, resembles *Hemophilus pertussis*. It is the cause of parapertussis, a disease which resembles mild pertussis from which it can be distinguished only by bacteriologic methods. (Synonym: *Bacillus parapertussis*.)

*H. parapertussis* was first reported by Eldering and Kendrick (1937) as a "group of cultures resembling both *B. pertussis* and *B. bronchiseptica*, but identical with neither," and was described by Bradford and Slavin (1937) as "an organism resembling *Hemophilus pertussis* with special reference to color changes produced by its growth upon certain media." In both instances, the organism was isolated from cases of suspected whooping cough. It is probable that it was observed in Copenhagen in 1933, though its clinical significance was not recognized at that time. Since its first recognition in America, it has been encountered in a number of our states and in Mexico. The demonstration of frequent occurrence of specific humoral antibodies among children by Miller in California and by Florsdorf in Philadelphia indicates that the infection may be a common one, easily overlooked. A small outbreak of the disease was observed by the author (1940) among a group of children attending a day camp.

The name *Bacillus parapertussis* was given to the organism by Kendrick (1938) who objected to its inclusion in the hemophilic group, although it is morphologically indistinguishable from *H. pertussis*. *H. parapertussis* grows well on Bordet medium, giving in 24 to 48 hours colonies which resemble those of *H. pertussis*, but are larger and

with the hemolytic zone more darkly discolored. It grows more readily than *H. pertussis* on plain agar and in liquid media, suitable for the latter organism. No report of its primary isolation on bloodless medium is as yet available.

*H. parapertussis* produces a brownish-black discoloration upon potato and a light brown discoloration upon Levinthal's medium. On egg-glycerin agar, a grayish-brown color resembling that of anchovy paste occurs. These color changes are helpful in distinguishing the organism from *H. pertussis* which produces no discoloration.

Like *B. bronchiseptica*, but unlike *H. pertussis*, *H. parapertussis* produces considerable catalase. Indol is not produced by growth in peptone water nor in tryptophane broth. Nitrates are not reduced. Milk is made alkaline more quickly and to a greater degree than by *H. pertussis*. Hydrogen sulfide is not produced by growth upon lead acetate medium.

*H. parapertussis*, when repeatedly subcultured over a period of several weeks upon chocolate blood agar, loses its hemolytic power and becomes weakly antigenic in rabbits.

*H. parapertussis* possesses common antigenic fractions with both *H. pertussis* and with *Brucella bronchiseptica* but is identical with neither (Chart 13). Cross agglutination between *H. parapertussis* and *H. pertussis* has been shown by Flosdorf (1942) to be caused by a common minor antigen.

*H. pertussis* produces a toxin similar to, but less potent than that of *H. pertussis* (Bruckner and Evans, 1939). Little is known concerning the general distribution or host range of the organism. The occurrence of multiple cases among siblings suggests that the disease is fairly communicable. Experimental infection is easily produced in mice by intranasal inoculation. In the mouse the experimental disease is characterized by a moderate leukocytosis and by pulmonary lesions resembling those pro-

duced by *H. pertussis* (Bradford and Wold, 1939).

The incubation period of the disease in man is from 6 to 15 days. The onset is similar to that of whooping cough, but may be more abrupt. The cough is less severe.

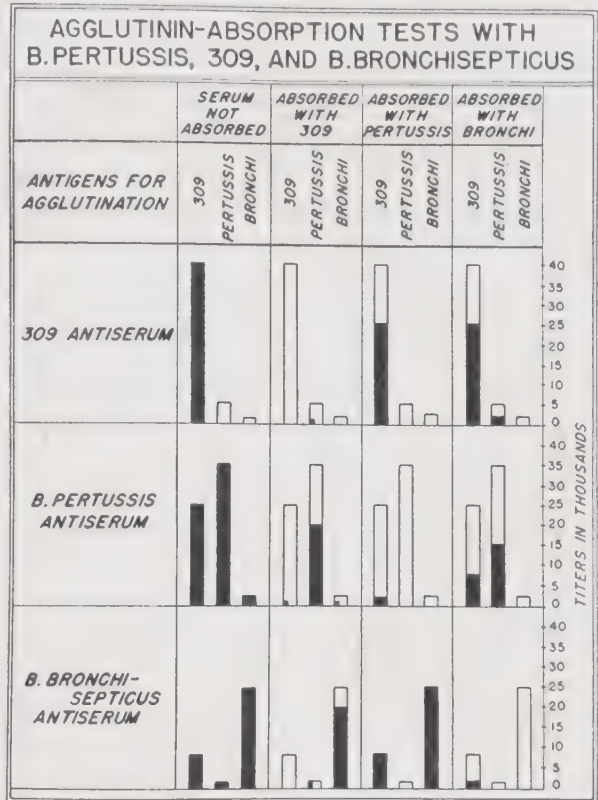


CHART 13. The antigenic relationship of *H. parapertussis* (309) with *H. pertussis* and with *Brucella bronchiseptica*. (Eldering, G., and Kendrick, P., 1938, *Bacillus parapertussis*: A species resembling both *Bacillus pertussis* and *Bacillus bronchisepticus* but identical with neither. *Journal of Bacteriology*, 35, 561-572.)

but is spasmodic, and is sometimes followed by a whoop. Vomiting is less frequent. The entire course of illness is from 1 to 3 weeks. The disease often resembles tracheitis. Complications are rare, but fatal pneumonia has been reported by Zuelzer and Wheeler (1946).

It is not known that one attack of parapertussis confers lasting protection. Second attacks have not been reported. An attack of parapertussis does not confer immunity



against pertussis, nor does an attack of pertussis confer protection against paraper-tussis. Humoral antibodies appear during convalescence, but nonspecific *H. pertussis* antibodies do not appear.

Treatment is symptomatic. The organism

is about 10 times more resistant to streptomycin than is *H. pertussis*, but has been observed to rapidly disappear from the upper respiratory tract following administration of this drug in the form of nasal drops.

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## 25

# The Meningococci

### INTRODUCTION

The meningococci (*Neisseria intracellularis*) are Gram-negative cocci, usually occurring in pairs, which ferment dextrose and maltose with the production of acid and which form nonpigmented colonies. In nature, they are only pathogenic for man and are the cause of epidemic cerebrospinal meningitis and cerebrospinal fever (Fig. 2H). (Synonyms: *Diplococcus intracellularis meningitidis*, *Neisseria meningitidis*, *Meningococcus*.)

### HISTORY

The early history of cerebrospinal fever is confused because of its resemblance to many other clinical syndromes in which the predominant features are central nervous system involvement or a rash. Thus, it was frequently termed "brain fever," "spotted fever" or "sinking typhus." In the early part of the nineteenth century, epidemics which are believed to have been of cerebrospinal meningitis were described in Switzerland, the United States and Sweden (Hirsch, 1886). Isolation of the causative organism by Weichselbaum, in 1887, permitted etiologic diagnosis based upon examination of the spinal fluid. The organism, termed originally *Diplococcus intracellularis meningitidis*, was subsequently isolated in the United States, Germany, England and France (Murray, 1929; Branham, 1940; Hedrich, 1931). With the ability to distinguish a specific type of meningitis due

to meningococci from other types of meningitis, there emerged a distinctive clinical and pathologic syndrome. As the performance of lumbar punctures and blood cultures became customary, it was found that meningococcal meningitis and cerebrospinal fever could occur either epidemically or sporadically. Occasionally, positive blood cultures were found without subsequent involvement of the central nervous system, and the term meningococcemia was coined. Postmortem examination often revealed metastatic lesions, other than meningitis, from which meningococci could be cultured, such as endocarditis, pericarditis, suppurative arthritis and epididymitis. More important, it was found that the organism could also be isolated from the nasopharynx of apparently healthy individuals. Often, the nasopharyngeal strains failed to agglutinate in antisera prepared with strains isolated from patients and hence were termed "parameningococci" (Dopter, 1909; Dopfer and Pauron, 1914). Subsequent experience showed that these parameningococci also occurred among clinical cases, and various systems for serologic classification were developed (Branham, 1940).

### MORPHOLOGY

The meningococcus, a member of the genus *Neisseria*, is a Gram-negative diplococcus, approximately 0.6 to 0.8 micron in

diameter. Individual cocci are ellipsoid, but usually they occur in pairs with the adjacent sides somewhat indented so that they appear reniform in shape. In older cultures, giant forms up to five times the size of the normal cell may be seen (Murray, 1929). Individual organisms vary in their ability to stain with either methylene blue or the Gram stain. These variations cannot be attributed entirely to differences in staining technic and are probably due to the rapid deterioration of the organisms, since meningococci contain a very active autolytic system. Many ghost forms are present in older cultures (Flexner, 1907). Tests for the presence of a capsule by direct capsular stains give equivocal results. However, addition of specific antiserum to certain cultures of meningococci recently isolated from patients reveals a swollen capsule; this has been observed only with strains of meningococci of Group 1 and Group 2a (Milner and Shaffer, 1946).

The colony is never pigmented and is not hemolytic. It is transparent, convex, glistening, with a smooth border, and of a creamy consistency. The regularity of this colony formation is so marked that when growth occurs upon a transparent medium, the colony may serve as a lens through which distant objects can be sharply focused. When many colonies are present on the medium, they often have a tendency to merge and form a mucoid streak of growth. A colony that can be pushed about on the medium or which fragments when touched with a platinum loop is almost never a meningococcal colony. The colony tends to lose some of its distinctive features when incubation is prolonged beyond 24 hours. The central portion becomes somewhat opaque and granular, while the periphery spreads and flattens with loss of the sharply defined round border. Occasionally, especially with freshly isolated strains, giant colonies are observed several millimeters in size. Strains which have been carried for prolonged pe-

riods on artificial media and some nasopharyngeal strains do not have the glistening mucoid appearance. Other important characteristics are also absent among such strains.

## GROWTH REQUIREMENTS

Meningococci have long been considered fastidious in their growth requirements (Murray, 1929). Growth is ordinarily not obtained with freshly isolated strains unless complex organic substances such as starch, pea powder, blood, serum, ascitic fluid or animal proteins are added to the usual media. The exact growth requirements have never been defined, but it is apparent that failure to grow is often due to the toxic action of amino acids, fatty acids or salts rather than to a nutritional deficiency (Frantz, 1943). By careful adjustment of the hydrogen ion concentration to a pH of 7.4 to 7.6 and by the addition of any of the materials enumerated above (pea powder, crude starch, or animal protein), which adsorb the toxic substances, growth has been readily obtained in the usual infusion media.

In fluid media, growth is not luxuriant and cultures frequently die within several days. A granular sediment is formed, and occasionally a pellicle is observed. The addition of dextrose or maltose to fluid media, while stimulating growth initially, shortens the life of the culture, probably because of the resulting acidity. The addition of agar to produce a semisolid or solid medium markedly enhances growth and prolongs the viability of the culture.

One of the most satisfactory media is the following starch casein hydrolysate agar, introduced in 1941 by Mueller and Hinton.

- A. 17 Gm. dry shredded agar  
500 cc. tap water  
Autoclave together at 15 lbs. for 15 minutes.
- B. 17.5 Gm. casamino acids technical (Difco)



300 cc. beef heart infusion (double strength)  
1.5 Gm. starch (Argo Laundry Starch) mixed into a paste with 10 cc. cold tap water  
90 cc. boiling water  
2 cc. phenol red (0.2 per cent aqueous)  
30 cc. 1.0 N sodium hydroxide  
75 cc. tap water

**Procedure.** Add casamino acids to heart infusion. The 10 cc. of starch paste is mixed with the 90 cc. of hot water, brought to a boil and then added to the casamino acids and heart infusion. The remaining ingredients are then added. (A) is then mixed with (B) and autoclaved at 10 lbs. for 10 minutes in 500 cc. lots. Pour plates with about 20 cc. of media per plate. These plates should be pink and the pH approximately 7.6.

Paramino benzoic acid may be added to solution B when antagonism of sulfonamides is desired. Sulfonamide drugs may be added to the hot media just before the plates are poured.

The medium does not require fresh blood or animal sera and is reproducible. Because it does not support the growth of streptococci and pneumococci, it permits primary isolation of meningococci from contaminated areas such as the nasopharynx. It is excellent for the maintenance of cultures in the laboratory and as a base to which carbohydrates may be added for fermentation tests. It has frequently been used to assay the antimeningococcal activity of the various sulfonamides because of its low content of sulfonamide inhibitors.

### BIOCHEMICAL ACTIVITY

The meningococcus is a strict aerobe. Dextrose and maltose are fermented with the production of acid but without the formation of gas. This property may not be apparent at first trial but will usually be observed upon repetition of the tests. With certain strains, the acidity may be only transient, and there is a subsequent reversal of pH. Lactose, sucrose and levulose are not fermented. The meningococci contain cytochrome oxidase which rapidly oxidizes dimethyl- or tetramethyl-paraphenylenediamine hydrochloride (McLeod et al.,

1934). This reaction is observed only with viable organisms. A similar oxidase is present in the other *Neisseriae*. Indole and hydrogen sulfide are not formed. Catalase is present.

It is unusual to obtain proliferation of recently isolated cultures at temperatures below 30° C. or over 40° C., and the optimum is between 35° and 37° C. Strains maintained on laboratory media for some time exhibit a greater temperature tolerance. Growth from small inocula is markedly enhanced when incubation is performed in an atmosphere of 5 to 10 per cent carbon dioxide—a fact of importance for primary isolation.

The meningococci contain an active autolytic enzyme system which is probably responsible for the swelling and the variation in staining properties noted in cultures more than a few hours old. Rapid dissolution of heavy suspensions of viable organisms can readily be observed upon the addition of alkali or upon incubation under toluol. The addition of potassium cyanide or heating to 65° C. for thirty minutes will inhibit this process (Flexner, 1907). Inactivation of the autolytic enzymes is often required for the satisfactory performance of agglutination reactions.

### SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

Meningococci are highly susceptible to various inimical agents. Drying, cold and the common germicides in low dilution readily kill them (Murray, 1929; Miller, 1942). Over 98 per cent of case strains are inhibited in vitro by 0.5 mg. per cent or less of sulfadiazine (Schoenbach and Phair, 1948a). Gramicidin, although chiefly active upon Gram-positive organisms, is lethal in a concentration of from 0.01 to 0.02 mg. per cent. Penicillin is inhibitory in a concentration of from 0.1 to 0.5 Oxford units per cc. (Miller and Bohnhoff, 1945). Streptomycin is active in a concentration of 1 to

40 units per cc., but tolerance up to 75,000 units per cc. is rapidly developed in vitro (Miller and Bohnhoff, 1947).

### IMMUNOLOGIC CLASSIFICATION

During the epidemic period of 1914 to 1918, extensive study of both case and carrier strains of meningococci by agglutination and agglutinin absorption reactions (Gordon, 1917; Murray, 1929) led to a classification of the group into 4 types, 1, 2, 3, and 4, which has achieved general acceptance in England and the Western Hemisphere (Branham, 1940). However, on the basis of clinical, epidemiologic, chemical, and serologic observations, it is believed impracticable to distinguish between Type 1 and Type 3 which are now classified jointly as Group 1. Type-2 strains are not homogeneous serologically and are therefore more appropriately designated as Group 2. A number of strains, formerly classified as Type 2 or Group 2, have recently been separately designated and are now referred to as Group 2a (Branham, 1942). Type 4 has not been isolated in the United States from clinical cases and is so uncommon that its classification seems unwarranted at the present time.

Many nasopharyngeal strains are so non-specific that they cannot be agglutinated in any of the specific sera. Many of them are agglutinated in polyvalent sera prepared by immunizing horses with mixtures of strains. At present, little clinical importance is attached to them, and they are classified as a "polyvalent" group.

Group 1 has been the most prevalent in the recent epidemics in the United States, while Group 2 has more commonly been isolated from cases occurring in interepidemic periods (Branham, 1940). The distribution of antigenic groups among more than a thousand case strains isolated from military personnel in 1943 was as follows: Group 1, 92.9 per cent; Group 2, 1.1 per cent; Group 2a, 5.8 per cent; Type 4, 0.0

per cent; polyvalent and unclassified, 0.2 per cent (Phair, Schoenbach and Root, 1944).

Group-1 and Group-2a strains readily give rise to specific antibodies when injected into animals, whereas most Group-2 strains require a prolonged course of immunization for the production of an adequate titre. Specific antisera cause capsular swelling with Groups 1 and 2a but not with Group 2. Similarly, the "halo" reaction is usually observed only with Groups 1 and 2a. The latter reaction is observed when a heavy localized inoculum on an agar medium containing specific antiserum is incubated for 48 to 72 hours; a ring of precipitate can then be seen about the growth. This precipitate has been attributed to the interaction of antibody with homologous specific antigen which diffuses into the medium (Petrie, 1933; Pittman et al., 1938).

### IMMUNOCHEMICAL ANALYSIS

The nucleoproteins of meningococci are antigenic and apparently account for the toxicity of the intact organism (Kabat, 1943). They are not specific for meningococci as they cross-react with antisera prepared with other *Neisseriae* and with pneumococci. A polysaccharide fraction (termed "C" substance) has been separated (Kabat, 1943). It is not specific for the meningococci but is common to all *Neisseriae*, pneumococci, and certain strains of Friedländer bacilli. The "C" fraction reacts with antibody against the nucleoprotein fraction described above. Both the nucleoprotein and the "C" fraction may play a role in immune reactions common to the genus *Neisseria* and related organisms. While these fractions have been designated as nucleoprotein and carbohydrate respectively, they have not yet been isolated as chemically defined entities.

A polysaccharide specific for Group-1 meningococci (Types 1 and 3 of Gordon and Murray) has been isolated from this



group (Scherp and Rake, 1935; Kabat, 1943; Branham, 1940; Kabat et al., 1944). It has been purified as the sodium salt of a nitrogen-containing polysaccharide acid and is identical in Type 1 and Type 3. The polysaccharide is not in itself a complete antigen but is probably responsible for the specific agglutination, precipitin and "halo" reactions of Group-1 meningococci. The specific protection afforded by Group-1 antisera has been closely correlated with their antipolysaccharide content (Pittman, 1938). However, in recent years, it has been shown that Group-1 horse, rabbit and chicken antimeningococcal sera contain considerable amounts of antibody which cannot be removed by absorption with either suspensions of Group-2 meningococci or purified preparations of Group-1 specific polysaccharide (Kabat et al., 1945). The residual antibody can protect mice against experimental meningococcal infection. The antigen of Group 1 responsible for its production is unknown.

Group-2 meningococci contain an antigenic protein fraction specific for that group. Removal from this fraction of a protein component yields a specific material ("kappa" substance) which is a carbohydrate-polypeptide complex. The "kappa" substance is not itself a complete antigen but appears to be correlated with Group-2 specific antibodies (reviewed by Kabat, 1943).

### PATHOGENIC PROPERTIES

Virulence among meningococci is an unstable quality difficult to appraise. Man is the only known natural host, and early observers believed that meningococci were essentially nonpathogenic for experimental animals since the minimal infective inocula are so large that they approach the lethal dose of heat-killed organisms (Murray, 1929). On the other hand, a fatal infection can be initiated in mice injected intraperitoneally with relatively few organisms sus-

pended in 2 to 5 per cent hog-gastric mucin. The mucin technic has been extensively used to assay the virulence of meningococci, the amount of protective antibody in sera, and the therapeutic activity of drugs. Mucin probably interferes with the normal phagocytic and possibly other immunologic defense mechanisms, thereby allowing the meningococci to establish a focus of infection from which they can be disseminated (McLeod, 1941; Olitzki et al., 1947). This artificially induced infection in mice differs so markedly from the natural disease that conclusions drawn from it may not be directly applicable to man. Embryonated hens' eggs can also be used to study the virulence of meningococci and the protective activity of drugs. Very small inocula introduced into the yolk sac of a ten-day-old embryo will initiate a fatal infection which, unlike the artificially induced infection in mice, has some characteristics in common with the disease in man. The above techniques have not demonstrated any distinct correlation between serologic grouping and virulence. On the other hand, rough, non-specific strains and those which have been transferred repeatedly for long periods of time on artificial media have low virulence.

The lethal activity of heat-killed or autolyzed cultures of meningococci has been attributed to an endotoxin assumed to be a nucleoprotein because of the method used in its preparation (Murray, 1929). The nucleoprotein fractions isolated by more modern methods are also quite toxic. Although true exotoxins specific for each of the meningococcal types have been described, their existence has not been convincingly demonstrated (Branham, 1940).

### IMMUNITY

The extent and mechanism of immunity to meningococcal infection are unknown. There is no reliable method for measuring the susceptibility of an individual to the disease, such as the Schick test for diph-

theria, and it is not even known whether or not one attack of the disease confers immunity. No successful immunizing procedure has thus far been developed.

The chief defenses of the host against meningococci appear to be phagocytosis and immune bacteriolysis. Bacteriolysis, as with other Gram-negative organisms, is mediated by specific antibodies plus complement. The blood of many normal animals appears to possess bactericidal properties against meningococci *in vitro*. Thus, Group-2 meningococci incubated in rabbit blood and Group 1 in guinea pig blood fail to multiply and are destroyed.

A discussion of the natural immunity of man to meningococcal infection immediately encounters a difficulty which is inherent in the meaning of the term "immunity." Although the number of clinical cases of meningococcal infection is small even in the most severe epidemic, the vast majority of individuals (often referred to as "carriers") harbor meningococci in their nasopharynx unassociated with overt clinical symptoms. If "immunity" is defined as resistance to clinical attack, then the natural immunity of man appears to be high. But if the localized nasopharyngeal infection (also termed "carrier state") is considered as the mild manifestation of a host-parasite relationship, then the natural immunity of man is extremely low. *In vitro*, the blood of a high proportion of the adult human population possesses bactericidal properties against meningococci. However, the results are so erratic and the variations with different meningococcal strains so great that there is little evidence that this property is a measure of immunity (Murray, 1929; Thomas and Dingle, 1943).

The degree of immunity resulting from clinical infection is unknown. The morbidity rate even during an epidemic period is so low (the annual rate rarely exceeds 4 per 1,000 individuals) that statistically the probability of observing a second attack is small. The situation is further complicated

by the existence of several specific meningococcal strains. The formation of antibodies as a result of disease, can be demonstrated by agglutination, mouse protection, quantitative precipitin reaction, complement fixation, and bactericidal technics (Kabat et al., 1945; Thomas et al., 1943; Mayer and Dowling, 1944). The formation of antibodies in the sera of known "carriers" has not been conclusively demonstrated. It seems, however, that meningococci undergo more rapid destruction when incubated in the fresh serum or blood of known carriers and that the presence of meningococci in the nasopharynx, although unassociated with clinical symptoms, serves as an antigenic stimulus (Rake, 1935). This will be discussed more fully under Epidemiology.

Antisera have been prepared by injecting rabbits, chickens, guinea pigs and horses with meningococci. These antisera contain agglutinins, precipitins and complement-fixing antibodies. When immunization is prolonged, the antisera formed become broader in reaction so that agglutination is no longer selective. When the strain employed for immunization has been maintained on artificial media through repeated passages, the antisera formed will not agglutinate many recently isolated strains except at higher temperatures (56° C.).

Numerous studies have indicated a correlation between the group specific antibodies and the protective value of antisera (Branham, 1940). However, while absorption with Group-1 specific polysaccharide reduces the protective content of the serum, absorption with the whole homologous organism is necessary for complete removal (Kabat et al., 1945). This suggests that some antigenic factor other than the polysaccharide and also specific for each of the serologic groups, plays some part in eliciting the production of protective antibody (Scherp and Rake, 1945).

Artificial immunization of the human has been attempted by means of vaccines, autolysates, modified toxins, and specific poly-



saccharide (Dingle and Finland, 1942). The results cannot be appraised in terms of protective antibody, and serologically the response has been poor (Kabat et al., 1944).

### CLINICAL DISEASE

The primary portal of entry of meningococci is the nasopharyngeal region. It was believed formerly that the organism then passed directly from the nasopharynx to the meninges via the lymphatics. However, the frequent isolation of meningococci from the blood stream both before and coincident with signs of meningeal involvement has led to the universal acceptance of the blood stream as the important route of meningeal infection. Infected thromboemboli are commonly observed in the capillary networks of the skin, choroid plexus and other organs and probably act as the foci from which meningococcal infection becomes established in the central nervous system and elsewhere (Murray, 1929).

Meningococcal infection may be considered as having three distinct stages. The first is a localized infection of the nasopharynx, the second is a septicemia (referred to as meningococcemia or cerebrospinal fever), and the third (or metastatic phase) is a purulent meningitis. Clinically, the disease may become arrested at any one of these stages, or the progression through the stages may be so rapid that they appear to coexist (Herrick, 1918).

In the first stage, the nasopharynx is inflamed; the conjunctivae are congested; and there may be a purulent rhinitis. The appearance is nonspecific, and the infection can be distinguished from a number of others only by isolation of the etiologic agent.

In the second stage, the patient presents the picture of general sepsis, with fever, chills, rapid pulse, malaise and often a mild arthralgia. The most distinctive feature is the appearance of irregular, dusky red spots or petechiae, each about 10 to 15 mm. in

diameter, in the skin and mucous membranes. The rash does not fade on pressure. In mild cases, it may be maculopapular and resemble that seen in typhus. Each petechial lesion persists for only a few days, leaving a small brownish stain as it fades. In severe cases, the petechiae may become hemorrhagic and assume a purpuric appearance. These vascular lesions, upon histologic examination, are found to be due to thromboembolic involvement of the capillaries. The organisms can be demonstrated in the fluid expressed by puncturing the lesions. This second stage is probably a true septicemia rather than a bacteremia. There may be massive infection of the blood and, occasionally, organisms can be seen on direct smear. This stage may be fatal in a few hours or may be of long duration with periods of remission and exacerbation. The fulminant type of infection with diffuse hemorrhages into the adrenals is usually termed the "Waterhouse-Friderichsen syndrome." Circulatory collapse and shock are the most prominent features.

The third stage occurs as a complication of the second. Metastatic complications such as endocarditis, purulent arthritis, or ophthalmitis are frequent in untreated cases, but the most common and dramatic complication is meningitis. The latter may be ushered in by a short prodromal period but often is sudden in onset and appears to coincide with the first symptoms of infection. It is similar in its symptomatology to other forms of purulent meningitis and is characterized by intense headache, emesis, stiff neck, positive Kernig and Brudzinski signs, and, in the more severe infections, by coma and convulsions. The rash, which may precede or accompany the meningitis, provides the chief clue as to the causative agent. In infants, frank meningeal signs are often masked and bulging fontanelles, gastrointestinal hemorrhage, convulsions or purpura provide a clue as to the nature of the disease. Herpes labialis is frequently associated with meningococcal meningitis.

## PATHOLOGY

The choroid plexus of the brain is swollen, congested and infiltrated with polymorphonuclear leukocytes. The blood vessels are thrombosed and erythrocytes are extravasated. The meninges are acutely inflamed in the earlier stages but later become thickened. A purulent exudate may be found in the lateral ventricles and along the cerebrospinal fluid pathway. It is present between the arachnoid and pia mater, about the base of the brain, and over the cortical sulci. Encephalitic involvement with focal collections of leukocytes, erythrocytes, and round cells about blood vessels is not uncommon. Meningococci may be present in the exudate and in the encephalitic foci. Similar inflammatory involvement of the spinal cord and of the surrounding pia-arachnoid is usually seen. In addition, petechial hemorrhages may be present in the skin and serous membranes. Metastatic lesions may be found on the endocardial and pericardial surfaces of the heart, in the joints, epididymis, lung and bone. Hemorrhage into the adrenals (Waterhouse-Friderichsen syndrome) has frequently been noted and its association with the fulminating cases has been mentioned above (Kinsman et al., 1946).

## DIAGNOSIS

The diagnosis of meningococcal infection can usually be confirmed bacteriologically, but a meticulous technic and a thorough understanding of the pathogenesis of the disease are required so that specimens be obtained from the proper sites at the optimal periods (Dingle and Finland, 1942; Branham, 1941). Early in the disease, blood cultures are positive in from 40 to 60 per cent of cases. Approximately 5 cc. of blood should be inoculated into 60 cc. of medium; it is also advisable to spread 0.1 cc. of blood over a starch-casein hydrolysate or blood agar plate in order to obtain an indication of the number of organisms. The cultures

are incubated at 35° to 37° C. in a moist atmosphere containing from 5 to 10 per cent carbon dioxide, and examined and subcultured daily for approximately seven days before being discarded. Identification of Gram-negative diplococci will be described subsequently. Occasionally, infection is so overwhelming that the diplococci can be seen in blood smears prepared for hematologic examination. More frequently, they can be seen in, and successfully cultured from, the fluid expressed by puncturing the petechiae in the skin.

Spinal fluid obtained by a lumbar puncture at the level of the fourth lumbar interspace is observed for its initial pressure, color and clarity. About 5 cc. is collected for a cell count, smear and immunologic tests, and a few cc. in a separate tube for chemical determinations. Cultures are obtained by allowing the spinal fluid to drop directly onto slants containing freshly prepared media and into a tube of broth. Some of the spinal fluid is collected in a sterile tube and incubated at 35° to 37° C. as soon as possible. It may yield positive growth when the former culture is negative. More recently, two new technics for the isolation of meningococci from the spinal fluid have been suggested. In one, mice are inoculated intraperitoneally with a spinal fluid mucin mixture (Sulkin, 1939). In the other, 8-day to 10-day embryonated hens' eggs are inoculated directly into the yolk sac (Blattner et al., 1943). These two methods have not yet been evaluated.

Smears prepared from the centrifuged sediment of the spinal fluid and stained with methylene blue reveal the typical diplococci extracellularly or within leukocytes. This examination should be made soon after the spinal fluid is withdrawn, since meningococci often disappear rapidly as a result of autolysis. If organisms are plentiful, direct typing by the addition of specific sera and examination for capsular swelling may be successful. A positive precipitin reaction is obtained at times when no organ-



isms are visible in the smear, by layering the supernatant fluid over monovalent anti-meningococcal sera. The precipitinogen in the spinal fluid is usually specific for the individual antigenic groups, and only rarely does the "C" polysaccharide present in all *Neisseriae* cause confusion. A positive precipitin reaction is usually found in association with the more severe infections (Alexander and Rake, 1937).

In meningococcal meningitis, polymorphonuclear leukocytes predominate in the purulent spinal fluid. When the disease is epidemic, a purulent spinal fluid, in which no micro-organisms are demonstrable, should tentatively be considered as meningococcal in origin. Spinal fluid sugar is reduced in this, as in all other pyogenic types of meningitis, and returns to normal as the infection subsides. Other chemical determinations such as spinal fluid protein concentration and colloidal gold curve only reflect the inflammatory character of the fluid.

Nasopharyngeal cultures are usually positive in clinical cases. In addition, a vast number of asymptomatic nasopharyngeal infections can be detected by cultures taken from this area. A sterile bent wire (preferably aluminum), with cotton wrapped about one end, is carefully placed behind the uvula and soft palate and thence inserted into the rhinopharynx taking care to avoid contamination by the bacterial flora of the mouth and throat. The swab is used to inoculate warm fresh solid media over a small area which is then streaked. Often, cultures of meningococci are obtained in almost pure culture. If necessary, the nasopharyngeal swab may be preserved in sterile defibrinated horse blood; agar cultures inoculated at a later period are entirely comparable with those directly inoculated and incubated (Schoenbach and Phair, 1948b).

Spinal fluid and blood usually yield cultures not contaminated with other species. Colonies suspected of being meningococci can be subcultured for identification by Gram stain and fermentation tests in media

containing dextrose, maltose and sucrose. Although meningococci usually ferment dextrose and maltose within 24 to 72 hours, the test may have to be repeated because of the erratic behavior of freshly isolated strains (Branham, 1941). Serologic identification is obtained by agglutination of the meningococci suspended in normal saline, preferably containing 0.1 per cent potassium cyanide to inhibit autolysis. The antigenic Groups 1, 2a or 2 can be determined through the use of specific sera. Resort to polyvalent horse serum is sometimes necessary especially with nasopharyngeal and interepidemic strains. When polyvalent horse serum is used, a test with normal serum in dilution of 1 to 50 should always be run in parallel to control nonspecific agglutination. Various agglutination technics have been employed. The suspension may be shaken at room temperature, incubated at 37° C. for 2 hours in a water bath, or heated to 56° C. for several hours and placed in the refrigerator overnight. More strains are agglutinated at the higher temperatures, but specificity is sacrificed. The rapid test tube agglutination at room temperature has been extensively used and found to be reliable and convenient. However, if a careful antigenic analysis is to be made, agglutination at 37° C. is preferable (Branham, 1940; Miller, 1944).

Other members of the genus *Neisseria* are differentiated by fermentation reactions, growth characteristics and agglutination. The gonococcus ferments only dextrose, *N. catarrhalis* ferments none of the carbohydrates, while *N. sicca* ferments all three. The latter two species are also characterized by growth at room temperature, brittle and rough-appearing colonies, and spontaneous agglutination in saline or low dilutions of normal horse serum. Various pigmented species of *Neisseriae* have been described, but the only strain of any importance is *N. flavescens*, which has been isolated from a small epidemic of meningitis in Chicago (Branham, 1930). It does not ferment any

TABLE 44. DIFFERENTIAL CHARACTERISTICS OF NEISSERIAE

ORGANISM	APPEARANCE OF COLONY AFTER 24 HOURS' INCUBATION	GROWTH ON PLAIN-NUTRIENT AGAR	GROWTH AT 22° C.	FERMENTATION			OTHER
				DEX-TROSE	MALT-OSE	SU-CROSE	
<i>N. intracellularis</i>	Round, smooth, glistening, translucent, colorless, creamy consistency	—	—	+	+	—	3 distinct antigenic groups serologically
<i>N. gonorrhea</i> ...	Similar to <i>N. intracellularis</i> . Smaller and more opalescent	—	—	—	+	—	
<i>N. catarrhalis</i> *	Smooth, glistening, translucent or firm, somewhat opaque and adherent. May be difficult to emulsify	+	+	—	—	—	Often agglutinates in normal horse serum or saline
<i>N. flavescens</i> ...	Yellow pigmentation when first isolated, otherwise similar to <i>N. intracellularis</i>	+	±	—	—	—	Homogeneous; distinct group serologically
<i>N. perflava</i> *...	Greenish-yellow pigmentation	—	—	+	+	+	Spontaneous agglutination in saline
<i>N. flava</i> *.....	Greenish-yellow pigmentation	±	±	+	+	—	Spontaneous agglutination in saline
<i>N. sicca</i> *.....	Small, somewhat opaque, wrinkled colonies, quite brittle	+	+	+	+	+	Spontaneous agglutination in saline and normal horse serum

\* These groups are so variable in their appearance and fermentative activities that it may be wiser to consider them together as *N. pharyngis* (Wilson and Smith, 1928).

of the carbohydrates mentioned. It is homogeneous serologically and can be identified by agglutination in its specific antiserum.

### TREATMENT

In 1906, Jochmann prepared an anti-meningococcal serum in horses and with it treated about 30 human cases of meningitis with encouraging results. Flexner (1913) reported a 50 per cent reduction of mortality in a series of 1,300 cases of meningitis by the use of antibacterial serum; he emphasized the importance of early treat-

ment. However, disappointing results were obtained by serum therapy during the epidemic of meningococcal meningitis in the United States in 1928. This failure led to further studies aiming at the preparation of improved antisera; antitoxic activity, mouse protection tests or titre of Group-1 antibody were used by different investigators as criteria in the preparation of the new sera (Branham, 1940; and Dingle and Finland, 1942). In spite of the marked variation in case fatality during different epidemics and among different age groups, it appears that serum therapy can reduce the mortality to



approximately 30 per cent as compared with from 60 to 75 per cent mortality in untreated cases (Jubb, 1943).

During recent years, sulfonamide therapy (especially with sulfadiazine) has supplanted serum therapy in meningococcal sepsis. The drugs are usually administered by mouth but, when the exigencies of the disease demand, may be given intravenously or intramuscularly. Treatment of experimental infections in mice with both antiserum and sulfonamides is superior to therapy with either serum or sulfonamides alone (Branham, 1938). However, there is no evidence at present that this combined therapy possesses any advantage over the use of the sulfonamides alone in the natural infection of man (Beeson and Westerman, 1943; Jubb, 1943).

Sulfadiazine was used extensively during the recent epidemic of meningococcal meningitis in the United States from 1942 to 1944. The mortality in the United States Army during this period was from 3 to 4.5 per cent, as contrasted with 40 per cent in a comparable group of American soldiers treated with antiserum in 1917 and 1918 (Thomas, 1943). In the civil population, when all age groups are included, the mortality with sulfonamide therapy has been between 10 and 20 per cent (Gover and Jackson, 1946).

Penicillin may be as satisfactory as sulfadiazine; systemic administration of the drug should be supplemented by the intrathecal route (Rosenberg and Arling, 1944). Recent observations that penicillin protects experimental animals from the lethal effect of meningococcal and gonococcal endotoxins suggests its use together with the sulfonamides in fulminant infections (Boor and Miller, 1945).

### EPIDEMIOLOGY

Although cerebrospinal fever occurs chiefly in epidemic waves, cases continue to appear in every large population group

in the intervening periods. Four distinct epidemics have been recognized in the United States since 1916. Each wave extended over several years and was followed by a period during which the incidence was low. The highest peaks were reached in 1917, 1929, 1936 and 1943. The last epidemic (1942-1944), with 16,491 cases for 1943 alone, was the most severe ever recorded by the Public Health Service (Gover and Jackson, 1946).

A seasonal cycle is quite evident during both epidemic and interepidemic periods. In the Northern temperate climate, the peak occurs between February and April, with a low rate from June to November. In tropical climates, the hot, dry periods are the seasons of greatest incidence.

The disease is world-wide in its distribution. It is more common among males; this difference was frequently attributed to the high incidence among military personnel. However, in the Detroit epidemic among the civil population in 1929, the ratio of males to females was two to one, a striking difference for which there is no adequate explanation (Norton and Gordon, 1930).

The age selection for cerebrospinal fever is similar to that noted for many endemic contagious diseases, such as measles, pertussis, scarlet fever and diphtheria. The attack rate among the very young far exceeds that for young adults and older age groups during both epidemic and interepidemic periods. The case fatality rate is also highest for the very young, although it again increases markedly for individuals more than 50 years of age. In Table 45 are presented the attack and case fatality rates observed in the province of Santiago, Chile, during 1942 when an estimated population of 1,290,000 experienced 3,586 cases and 553 deaths (Horwitz and Perroni, 1944).

Cerebrospinal fever is an important military disease. Almost every country experienced an outbreak of it coincident with military and industrial mobilization during World War II (Gover and Jackson, 1946).

TABLE 45. CASES OF MENINGOCOCCAL MENINGITIS, AGE SPECIFIC ATTACK RATE AND CASE FATALITY IN THE PROVINCE OF SANTIAGO, CHILE, FOR THE YEAR 1942 \*

AGE GROUP	NO. OF CASES	ATTACK RATE PER 100,000 POPULATION	CASE FATALITY (PER CENT)
Under 1 yr.	309	820	33.0
1- 4	770	620	24.6
5- 9	612	496	13.3
10-14	541	471	5.7
15-19	384	262	0.8
20-24	245	166	0.7
25-34	355	152	1.0
35-44	215	135	1.5
45-54	93	86	1.5
55-64	34	62	29.4
65+	22	58	54.6
Unknown	6	...	0.0
Total	3,586	278	15.4

\* Computed from data in Table 2 of Horwitz and Perroni, 1944.

In military personnel the incidence of the disease is highest in new recruits. An individual entering upon military service comes in intimate contact with a large number of persons assembled from different geographic areas, and increased exposure to many infectious diseases makes it worthwhile to consider military personnel with respect to their length of service—conveniently referred to as “environmental age.”\* During 1942 and 1943, 66 per cent of the cases of cerebrospinal fever in the Army occurred among personnel with 3 months or less of service (Sartwell and Smith, 1944). Among military personnel, therefore, environmental age appears to be analogous to chronologic age in the civil population with respect to the incidence of cerebrospinal fever.

A small percentage (2 to 5 per cent) of the normal population harbor meningococci in the nasopharynx during interepidemic periods. During epidemic periods, this rate

may reach 70 or 80 per cent, and organisms of the specific epidemic strain become prominent in the meningococcal flora of the nasopharynx. A study conducted among Army personnel revealed the presence of meningococci in the nasopharynx of 40 per cent of the group at each culture period. Moreover, 92.9 per cent of the same group of men were found positive at some time during the 3-month period of observation when examined 3 times weekly. This study was limited to meningococci specifically agglutinable by serum of each of the 3 main groups. Group-1 organisms, which had been responsible for all clinical cases, were isolated from the nasopharynx of 53.5 per cent of apparently healthy individuals during this period. The average duration of nasopharyngeal “infection” was approximately 3 weeks. These results make it clear that the probability of escaping contact and infection with meningococci is exceedingly small (Phair and Schoenbach, 1944). Nevertheless, clinical cases are relatively infrequent and occur chiefly among new recruits. It appears, therefore, that the vast majority

\* “Environmental age” is defined as the period of time during which an individual is subjected to or exposed to a specific environment. The expression is applicable, not only to military life, but to residence in institutions, geographic areas, etc.



of individuals acquire resistance to clinical disease as the result of inapparent infections. Although the nasopharyngeal "infection" rates are considerably lower in the civil population and during interepidemic periods, it appears possible that inapparent infections with resultant resistance are phenomena of wide occurrence and might explain the age selection, both chronologic and environmental, of meningococcal meningitis.

It is not known whether infection with any one serologic group of meningococci confers protection against clinical disease caused by a different serologic group. It should be noted in this respect that an individual can harbor a specific antigenic strain of meningococci in the nasopharynx for a period of time, then become negative on culture, and later harbor again either the same or a different antigenic strain (Phair and Schoenbach, 1944).

### CONTROL MEASURES

Methods of control of cerebrospinal fever are based upon the assumption that meningococci maintain themselves in a human population through inapparent infections, usually termed the carrier state. The clinical case usually acquires the organism from a "carrier" and only occasionally from another case. Isolation of patients can therefore play only an insignificant role in the control of the disease. Avoidance of overcrowding and provision for adequate ventilation may reduce the risk of infection. Isolation of all carriers in a population is impossible because of their large number and of the difficulty in identifying them. To effect control, therefore, in the absence of an immunizing agent, it is desirable to eliminate meningococci from the entire group under consideration.

Meningococci can be eradicated from a delimited group by the administration of 2 to 6 grams of sulfadiazine (Kuhns et al., 1943; Phair and Schoenbach, 1944). Small

doses of the drug have halted the spread of meningococcal meningitis without significant undesirable sequelae, particularly among controlled populations such as exist in military organizations and in institutions. In 1943, Kuhns treated 15,000 troops with 4 to 9 grams of sulfadiazine over a period of 2 to 3 days during an epidemic of meningitis. In the subsequent 8-week period of observation only 2 cases of the disease occurred in contrast with 40 cases among 18,800 untreated controls (Kuhns et al., 1943). In 1944 to 1945, one-half to one gram of sulfadiazine was administered daily to naval recruits for a period of 7 weeks in order to prevent streptococcal respiratory disease. Among 600,000 men thus treated, only 5 cases of meningitis were noted, 3 of which occurred within the first 24 hours of chemoprophylaxis. During the same period, 146 cases were observed among a smaller group of untreated individuals (Cheever, 1945). Chemoprophylaxis, once terminated, does not confer freedom from subsequent infection; infection among the treated group may again reach the level in the general community, and more rapidly the higher the degree of re-exposure.

Enthusiasm concerning chemoprophylaxis must be tempered by caution because of the possible effect of the sulfonamides on both host and parasite. Certain individuals are hypersensitive to these drugs, approximately 0.05 per cent in the case of sulfadiazine, and prophylactic administration may sensitize an additional number. With respect to the parasite, the small dosages of sulfonamides usually employed for prophylaxis may modify the etiologic population by favoring the survival of drug-resistant strains. This has been a common experience whenever the drug has been administered over a long period of time for the prevention of infections due to streptococci, pneumococci, gonococci and undifferentiated *Neisseriae*. Although eradication of meningococcal infection can be accomplished by chemoprophylaxis of short duration without causing

the appearance of resistant strains, the potential danger nevertheless exists. Chemoprophylaxis should therefore not be em-

ployed routinely for the prevention of meningococcal infections without careful consideration of the possible risks.

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## 26

# The Gonococci

### INTRODUCTION

The gonococcus (*Neisseria gonorrhoeae*) is a Gram-negative coccus which is the type species of the genus *Neisseria* (family *Neisseriaceae*) to which belong also the meningococcus and several nonpathogenic inhabitants of mucous membrane. It is the causative agent of gonorrhea (known in the vernacular as "clap" or "strain") and of ophthalmia neonatorum (Fig. 2G).

### HISTORY

Unmistakable references to gonorrhea are contained in the very early Chinese writings and in the Bible, and some writers consider that the origin of the Hebrew rite of circumcision may have had a bearing upon the prevention of balanitis, a frequent complication of gonorrhea. Galen in A.D. 130 first employed the term gonorrhea, translated as "flow of seed." During the late Middle Ages gonorrhea was thought to be a manifestation of syphilis. This concept gained support from the classic error of John Hunter, who, as late as 1767, misinterpreted the syphilitic infection which he contracted by self-inoculation with pus from the urethra of a patient supposedly infected with gonorrhea. Ricord in 1830 properly delineated the two diseases. In 1879, Neisser identified the causative organism which he called gonococcus and which was cultivated several years later by Bumm on artificial media. Finger, Ghon, and Schlagenhauser

published in 1894 a description of the histopathology of the disease based on the study of post-mortem material obtained from patients artificially infected in the terminal stage of other diseases. The disease has attained special prominence during the past three decades from the points of view of bacteriology, public health and social hygiene. This interest possibly reached its peak in 1943 when the very striking susceptibility of the *N. gonorrhoeae* to penicillin was demonstrated.

### MORPHOLOGY

The gonococcus appears in the exudate of acute gonorrhea as a diplococcus with contiguous sides, flattened or slightly concave, resembling a pair of kidney beans and measuring from 0.6 to 1.0  $\mu$  in diameter. It does not possess spores, true capsules or flagella. It is readily stained by aniline dyes and is Gram negative. Ordinary optical methods do not reveal any intracellular differentiation.

Cells growing in laboratory media differ somewhat in appearance from those seen in pathologic material. Cultures consist predominantly of single cells and small clumps but also frequently exhibit irregular-staining "giant" forms. Cellular morphology is progressively altered by contact with penicillin both in vitro and in vivo. Smears of exudate taken during the first four or five hours of



penicillin therapy show gonococcal forms that progressively enlarge and decrease in stainability until none can be detected. Similar morphologic changes are not observed during sulfonamide therapy.

### CULTIVATION

Primary cultivation of the gonococcus on laboratory media is difficult not only because the organism is fastidious in its growth requirements, but also because it is exceedingly susceptible to the toxic effect of a variety of substances commonly present in ordinary media. The organism grows best under aerobic conditions, at pH 7.2 to 7.6, at a temperature of 35° to 36° C. Some strains do not grow satisfactorily at 37.5° C., and in general growth stops below 30° C. or above 38.5° C. Most strains require an atmosphere containing from 2 to 10 per cent CO<sub>2</sub> to initiate development. Although gonococci grow well on the moist surface of solid media containing 1.2 to 1.5 per cent agar, excessive moisture as produced by syneresis of the agar is undesirable, especially for primary isolation, because it favors other bacteria and especially spreaders, which readily overgrow the more slowly growing gonococcus. Satisfactory growth is obtained on agar media consisting of meat infusion, peptones, glucose, buffered with phosphate and enriched with plasma and hemoglobin or whole blood. Increased yields can result from the addition to the media of yeast and liver concentrate which supply glutamine and cocarboxylase (shown by Lankford and Snell in 1943 to be essential for 10 to 15 per cent of gonococcus strains). Glutathione has also been found by Gould (1944) to be an essential growth factor for certain strains. As mentioned earlier, many components of media exert an inhibiting effect on the growth of gonococcus. Thus, certain amino acids occur in peptones in concentration sufficient to be somewhat toxic, but this effect can be markedly reduced or abolished by heating the medium

after addition of the blood (chocolate agar). Ley and Mueller (1946) have shown that agar also can exert an inhibiting effect on the growth of certain strains of gonococci and that the effect can be counteracted by the addition of charcoal or starch to the medium. The inhibitory substance appears to be a fatty acid. Since the gonococcus does not metabolize starch, and since starch becomes ineffective after hydrolysis, it appears that the beneficial effect of starch as well as of charcoal is due to the fact that they can adsorb the toxic agent; serum appears to play a similar role.

Media can be rendered more selective by the addition of certain dyes like Nile blue A or crystal violet in concentrations which are sufficient to inhibit many other bacterial species, but which do not affect the growth of the gonococcus. Advantage has been taken of all these different facts in certain ready prepared commercial media, like Bacto Proteose No. 3 hemoglobin medium with supplements, which are readily available and convenient for the isolation of gonococci from pathologic material.

After 48 hours' incubation, the primary gonococcus colony appears translucent, raised, finely granular, slightly convex with lobate margins. It is often mucoid and varies in size from punctiform to 5 mm. in diameter, depending on the medium and the crowding of the plate. Although ordinary agar cultures die within 3 to 4 days unless transferred, agar-slant cultures kept at 35° C. remain viable for a considerable period of time if covered with sterile paraffin oil.

Glucose is the only sugar fermented by the gonococcus, with the production of acid, but no gas; its failure to ferment maltose differentiates it from the meningococcus. The fact that the gonococcus produces indophenol oxidase has been made use of as a diagnostic test. When 1 per cent aqueous solution of p-aminodimethyl anilide monohydrochloride is added to an agar growth the colonies turn pink, then purple. The

reagent kills the organisms within a few minutes but does not modify their morphologic and staining characteristics. The oxidase reaction is often negative in media containing 1 per cent glucose; with the addition of neutralized reagent it can be rendered positive (Bucca, Thayer and Schubert, 1947). All members of the genus *Neisseria*, as well as certain bacteria and yeasts occasionally found in the flora of the urethra and cervix, can also produce indophenol oxidase. Nevertheless, positive oxidase reaction, coupled with typical colonial characteristics and the presence of diplococci resembling the gonococcus, constitutes presumptive cultural evidence which should be confirmed by sugar fermentation.

We have already mentioned that under ordinary conditions, the gonococcus dies rapidly in agar cultures. It is quickly killed by drying, sunlight and ultraviolet light. At 42° C. death occurs within a few hours in vitro (Carpenter et al., 1933). Moist heat at 55° C. kills the gonococcus in a few minutes. Phenol, bichloride of mercury and silver compounds are very effective disinfectants. Although the gonococcus is susceptible to sulfonamides, the range of susceptibility is much wider than in the case of penicillin, to which drug the organism is extremely susceptible. Moreover, specific resistance can be induced more readily to sulfonamides than to penicillin by culturing the organisms in increasing amounts of the respective drugs. Penicillin X appears to be more effective in vitro and in vivo than the commercial product containing predominantly G penicillin. Up to the present time no marked difference in mean penicillin susceptibility has been observed between strains isolated from cases of therapeutic failures and those isolated from patients successfully treated with the drug. The gonococcus is also susceptible to the action of streptomycin in vivo and in vitro, but induced resistance to this drug is rapidly acquired in vitro (Miller and Bohnhoff, 1946).

Finally it may be mentioned that the

gonococcus is more soluble than the meningococcus in dilute NaOH (within one minute) whereas other *Neisseria* are practically insoluble in this reagent.

Although different colony types of growth of gonococcus can be recognized, Mahoney et al. (1946) were unable to correlate colonial type and pathogenicity by injecting the organism into human volunteers.

The toxicity of gonococcus appears to be entirely due to an endotoxin which is heat resistant. Mice can be protected against its lethal effect by the injection of large doses of crude penicillin preparations. Other chemical components separated from the gonococcus have not so far proven of any practical significance in the development of tests useful in the diagnosis of gonorrhea. Failure to detect significant levels of antibodies by agglutination, precipitation, bactericidal, complement-fixation and allergy tests may be traced perhaps to the fact that the infection stimulates little antibody formation because of its local character.

## CLINICAL COURSE

The onset of the disease in men is usually sudden and is characterized by a superficial inflammation of the mucous membrane of the anterior urethra, accompanied by more or less profuse purulent discharge with pain and urgency on urination. The inflammatory process tends to progress posteriorly, involving the membranous urethra, the prostate seminal vesicle and the epididymis. When not treated by chemotherapy, the disease exhibits a tendency toward chronicity with minimal symptoms and frequent acute exacerbations, the "gleet" of previous years. This has practically disappeared since the advent of penicillin treatment.

In women, the infection involves first the urinary meatus and adjacent structures together with the urethra. Symptoms of pain, frequency and purulent discharge may be severe or of a minimal character. The process tends to recede from the urinary



tract and to involve the mucous membranes of the cervical canal, the cardinal sign being a profuse mucopurulent discharge. Progression to the fallopian tubes with the production of pelvic inflammatory disease of varying severity may ensue. Residual damage to the tubal structures may give rise to sterility and may require surgical intervention for relief of symptoms.

Blood-stream infection and the involvement of the endocardium with the production of vegetations on the valve leaflet have been reported, although instances in which the invading organism has been satisfactorily identified as *N. gonorrhoeae* by culture methods have not appeared in recent literature.

*N. gonorrhoeae* is responsible for ophthalmia neonatorum, an inflammation of the eye of the newborn resulting from infection during passage through the birth canal. The infection, which appears several days after birth, is always serious, frequently violent and destructive to the ocular structures, and is credited with being responsible for 12 per cent of all blindness. The superficial vessels become engorged, the conjunctiva markedly swollen, and a profuse purulent discharge develops. Ulceration of the cornea may follow, with possible extension of the process into the anterior chamber or, in some instances, to all of the ocular structures. Healing of the corneal lesions may give rise to opacities of varying magnitude with consequent loss of vision.

### DIAGNOSIS

Gram-negative intracellular diplococci in the stained exudate from a suspected gonococcal infection strongly suggests the diagnosis of gonorrhea. The intracellular position of the gonococcus is a common finding in acute gonorrhea, but in very early or chronic infection the organisms may be found only extracellularly, frequently as a single coccus, or it may even be impossible to demonstrate their presence microscopi-

cally. In men the diagnosis based on the characteristic clinical symptoms can usually be confirmed by the finding of the intracellular, Gram-negative diplococcus in pus cells in the urethral discharge and preferably by positive identification of *N. gonorrhoeae* by culture technic including the determination of the sugar-fermentation reactions. Trauma or the introduction of mechanical and chemical irritants into the urethra may give rise to an inflammatory process having some of the characteristics of gonorrhea but which can be differentiated by negative bacteriologic findings. Similarly, a condition designated as "nonspecific urethritis" is infrequently encountered. So far as is known, no microbial agent other than *N. gonorrhoeae* has the capacity to produce consistently acute urethritis in the human.

Gonorrhea in women may vary in intensity from a circumscribed and almost symptomless inflammatory process to widespread disease involving the mucous membranes of the urinary and birth canal structures, including the peritoneal covering of the adnexa. Laboratory diagnosis is more difficult than in men. Stained smears are of value in early infections when typical intracellular organisms may be found in material from the urethra or cervix. As the age of the infection advances the value of the spread finding decreases while that of the culture method increases. The relative reliability of the two methods has been studied in incarcerated prostitutes by Van Slyke et al. (1942). Specimen material was collected from each of 140 patients with clinical gonorrhea and positive culture findings. Stained preparations were prepared from each individual in triplicate and the slides examined by three experienced microscopists who reported as positive only 88, 47 and 40 of the specimens, respectively. Although the culture method is more reliable, it has also distinct limitations as practiced at the present time. In three laboratories employing identical technical methods and testing samples of identical mate-

rial, complete agreement could be obtained in only 75 per cent of patients known to be infected.

It should be pointed out in this connection that in chronic gonorrhea culture findings may spontaneously reverse from positive to negative without any accompanying change in the clinical status of the infection (Mahoney et al., 1942).

Complement-fixation tests are not sufficiently reliable to be used in diagnosis. Employing the Price and Kolmer methods with single and multiple strain antigens, Van Slyke et al. (1942) observed many instances of clinical gonorrhea with positive cultures but negative complement fixation.

Difficulties inherent in the isolation of the gonococcus are increased when the clinician fails to exercise care in securing exudates for cultivation. This is especially true of specimens obtained from women, since the bacteria present in the flora of cervical exudate may overgrow the gonococcus. Inoculations from gonorrheal exudate were found by Schubert, Bucca and Thayer (1947) to be most successful when made in the clinic directly on the agar surface and incubated immediately. When this is not possible, the swab containing exudate should be placed in a small amount of broth and taken to the laboratory.

Specimens of blood, synovial fluid and spinal fluid are best cultivated in shallow layers of ascitic fluid broth in an atmosphere containing from 2 to 10 per cent CO<sub>2</sub>. Urine specimens should be centrifuged and the sediment inoculated on agar medium. Prostatic fluid may be directly inoculated on agar or centrifuged in a small amount of urine and the sediment inoculated on agar. Using mailed cultures 24 hours old (which are not so satisfactory as those cultured immediately after collection), Usher and Stein (1945) were able to detect twice as many gonococcic infections as were found by microscopic examination of the stained smear.

For more detailed information concerning

the cultural method of isolating and identifying *N. gonorrhoeae* the student is referred to the following sources: Carpenter (1945), Thayer, Schubert and Bucca (1947) and the procedure for isolation and identification of the gonococcus, Venereal Disease Graphic 84.

In general, because of the characteristic clinical symptoms, presumptive cultural evidence of gonococcal infection (unconfirmed by sugar fermentation) is more acceptable in the diagnosis of gonorrhea in the male than in the female. In the female, where clinical evidence is of little value, a negative smear report coupled with presumptive cultural evidence may lead to as many as 16 per cent false-positive diagnoses. For example, Thayer (1943) cultivated oxidase-positive colonies of various micro-organisms from 36 per cent of 548 normal women. In 3.3 per cent the cultures were *Neisseria* other than the gonococcus, and in 12.6 per cent the organisms were morphologically and tinctorially so similar to the gonococcus that sugar-fermentation tests were necessary to rule out this organism.

## TREATMENT

Before the era of chemotherapy, treatment consisted of the local application of antiseptic and astringent solutions by means of injections, irrigations and instillations, and of attempts to alkalinize the urine. All these methods are of questionable efficacy. The use of sera, vaccines and culture filtrates has also proven to be of very limited, if any, value. Artificial hyperpyrexia, based upon the marked heat lability of gonococci, has been practised.

When the sulfonamide compounds and, subsequently, penicillin became available, the therapy of gonorrhea passed from the province of urology to that of chemotherapy.

At the present time the treatment of choice utilizes penicillin in an aqueous solution or incorporated in the peanut-oil-



beeswax vehicle which was developed by Romansky (1944). Repository preparations containing from 200,000 to 300,000 units are sometimes employed for one-injection therapy. With either type of therapy four per cent of treated individuals exhibit residual symptoms or a recrudescence of symptoms and require additional therapy using twice the amount of penicillin used in the initial therapy. A third course of treatment may be required in some instances. The need for local therapy, prostatic massage or artificial hyperpyrexia is very seldom encountered. Ophthalmia neonatorum is only rarely encountered at present, and its response to penicillin equals that of a urethral infection.

Up to the present there is no evidence of acquired resistance of *N. gonorrhoeae* to penicillin. Moreover, careful observation has failed to show the production of carrier states, characterized by the harboring of virulent organisms in the structures of the urinary tract of patients who are otherwise asymptomatic.

Adequate criteria of cure in men consist of complete freedom from clinical evidence of the disease and at least 3 negative cultures taken at weekly intervals following the completion of the treatment. The material for culture should be obtained from urine sediments. Expression of the urethral glands by gentle massage of the canal over a sound and prostatic massage may be employed, although the actual value of these procedures has not been demonstrated. As already mentioned, the tendency of the infection to produce asymptomatic carrier states is not great and most, if not all, patients harboring the *N. gonorrhoeae* will sooner or later display clinical evidence of infection. In women repeated cultures of carefully selected material from the cervix and urethra offer the only available means for detecting residual infection. A minimum of 3 negative cultures should be obtained. As a precautionary measure in both men and women, unprotected sexual exposure

should be interdicted for a period of 3 months after the criteria of cure have been satisfied.

An exceptional circumstance is presented when gonorrhea and syphilis are contracted concurrently. The gonococcic infection, because of its short incubation period, becomes evident while the syphilitic infection is still in the preclinical stage. Penicillin therapy directed toward the cure of gonorrhea probably may not be of sufficient intensity or duration to eradicate an oncoming syphilitic infection, the development of which may be so altered or masked that it will escape early recognition. Hence, all patients who have received penicillin treatment for gonorrhea should have monthly serologic tests for syphilis for four months after the completion of treatment.

## EPIDEMIOLOGY

The distribution of gonorrhea is worldwide; instances of racial immunity have not been recognized. The popular belief that an infection contracted from a member of a certain race or in certain geographic areas is more severe or more resistant to treatment than others has no factual basis. The disease is essentially venereal in nature, requiring intimate contact for transmission. The role of intermediary objects in transmission is inconsequential.

Individuals vary greatly in their resistance to infection. Mahoney et al. (1946) inoculated a large number of male volunteers by instillation of massive doses of cultures into the urethral canal. In a total of 245 experimental exposures, typical clinical disease with confirmatory laboratory findings was produced in only 83, or 33.8 per cent. In the remainder, a train of irritative symptoms of varying severity that persisted for periods ranging from several hours to two days was followed by a return to normal. The relatively low incidence of the infection among professional prostitutes may be the manifestation of such natural

resistance. In culture studies carried out over several years, Van Slyke et al. (1942) found approximately 20 per cent of incarcerated prostitutes and other sex offenders to be infected.

Specific vulvovaginitis is a form of gonococcal infection once thought to be capable of spreading in epidemic fashion through the population of girls' schools, infant hospitals, and infant asylums, and to be due to the use of common utensils and carelessness in nursing care. Cohn and Adler (1940) were able to demonstrate that: (1) vulvovaginitis due to *N. gonorrhoeae* is relatively rare, (2) that intermediary objects play a minor role in transmission, (3) that epidemics of specific vulvovaginitis probably do not exist, and (4) that in authentic infections a survey of circumstances usually reveals sex contact.

#### PROPHYLAXIS AND CONTROL MEASURES

The methods which have been used in the past for the control of gonorrhea have not been satisfactory and probably not effective. This may be attributed to the inadequacy of case reporting, extreme difficulty of diagnosis (especially in women), the fact that one infection does not produce an immune response sufficient to protect the patient against reinfection, and, up to recent times, the lack of a treatment suitable for mass therapy. Although penicillin therapy has not been in general use long enough to permit an unqualified conclusion, it appears likely to modify greatly the epidemiology of gonorrhea. Under present circumstances, the treatment reduces the period of infectiousness from months or weeks, as was

usual when local forms of treatment prevailed, to a matter of hours, thus decreasing enormously opportunities for transmission. This influence can hardly fail, eventually, to bring about a marked decline in the prevalence of the disease.

Health department practice calls for: (1) efficient case reporting in order that the health organizations be informed as to prevalence and distribution of the disease, (2) the providing of adequate treatment facilities, (3) the investigation of all sources of infection and the observation of individuals sexually exposed to a known infected person, and (4) the conduct of such educational efforts as the situation in any given community may require, both as regards the disease itself and the broader lines of social hygiene and sex.

Mechanical prophylaxis by means of a condom offers the only type of protection upon which reliance can be placed. In the male, transmission is effected by the contamination of the very distal portion of the urethral mucosa. On hypothetical grounds, any chemical agent capable of destroying *N. gonorrhoeae* while the organisms occupy a vulnerable position on the surface of the mucous membrane should serve as an effective prophylactic. There is no acceptable evidence, however, that the commercial chemical prophylactic preparations or those utilized in military organizations have any appreciable degree of effectiveness. In women, mechanical cleansing with soap and water or with mild antiseptic solutions offer a theoretical, but unconfirmed, source of protection. The use of penicillin or the sulfonamide compounds as prophylactics has not been adequately studied.



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## 27

# The Spirochetes

### INTRODUCTION

The *Spirochaetacea* are spiral organisms presumably devoid of flagellae, but which are nevertheless actively motile; the large majority divide by transverse fission. The six genera into which they are divided differ widely in their morphologic characteristics, growth requirements, distribution in nature, and pathogenicity. Organisms of the genus *Spirochaeta* have a central elastic filament around which the protoplasm is wound spirally. They are large, flexible and undulating, the type species *S. plicatilis* measuring 200 to 500  $\mu$  in length. These saprophytic organisms grow best at approximately 20° C., and are commonly found in sewage and other contaminated water. The genus *Saprosira*, also saprophytic, includes a group of tubular organisms wound in spirals, so that the cross section is circular. The *Cristispira* have a thin, flat ridge or *crista*, which runs spirally the length of the organism. The body itself is divided by septa. These organisms are parasitic in molluscs.

Three genera are pathogenic for man: (1) *Treponema*, of which *T. pallidum* is the cause of syphilis; *T. pertenue*, the cause of yaws; and *T. carateum*, the cause of pinta; (2) *Borrelia*, several species of which cause relapsing fever; and (3) *Leptospira*, several species of which cause spirochetal jaundice (Weil's disease).

Much confusion has been caused by the use of the term *spirochete*, and the generic classification *Spirochaeta*, for any spiral organism. Further, because of the lack of precise differential criteria between treponemata and borrelia, many organisms have been given both names. In consequence, the terms *Spiro-*

*cheta recurrentis*, *Treponema recurrentis*, *Borrelia recurrentis*, and *Spironema recurrentis*, have all been applied to the same organism; and the terms *Spirocheta pallida* and *Treponema pallidum* are used almost interchangeably.

### TREPONEMA PALLIDUM AND SYPHILIS

#### HISTORY

Syphilis appeared in western Europe in epidemic form in the year 1492. It is generally believed to have been acquired by Columbus' crew from the Indians, and brought by them to Europe. The extraordinary virulence of the disease at that time appears clearly in some of the early descriptions, and one may speculate as to whether the present relatively mild course of the disease in its early stages reflects a change in the organism or an acquired resistance in the general population. The causative organism was identified in 1905, when Schaudinn and Hoffman demonstrated its presence in the primary lesion and in the adjacent lymph glands of syphilitic patients. Noguchi and Moore subsequently found the organisms in the cerebral cortex of patients dying with general paresis, and thus proved that syndrome to be, as had long been suspected, a late manifestation of syphilitic infection.



## MORPHOLOGY

*Treponema pallidum* is a slender, spiral organism measuring 5 to 20  $\mu$  in length, and 0.2  $\mu$  in thickness (Fig. 28). Its 6 to 14

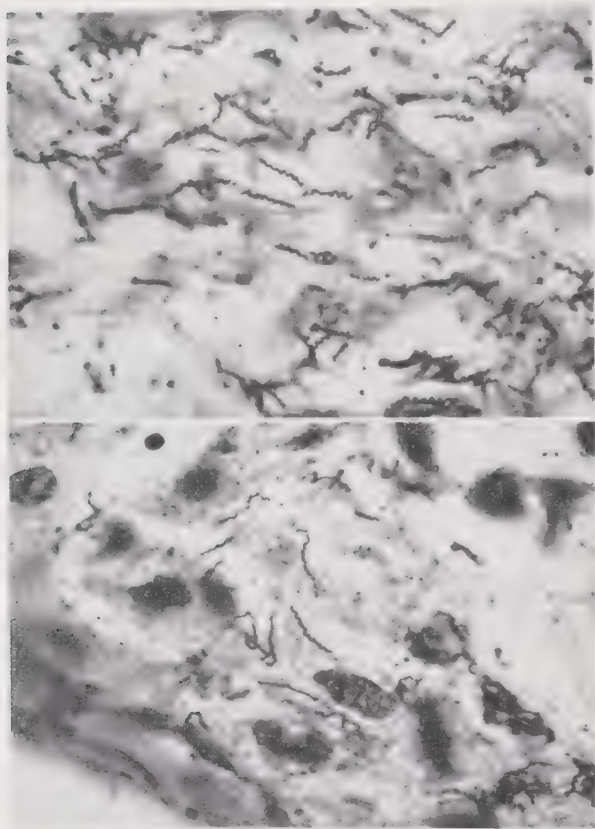


FIG. 28. (Top) Congenital syphilis of the lung. *Treponema pallidum* demonstrated by Levaditi's method. (Smith, L. W., and Gault, E. S., 1942, Essentials of Pathology, ed. 2, New York, Appleton, Fig. 121.)

FIG. 29. (Bottom) *T. pertenuis* of the skin; secondary yaw. Oil immersion photomicrograph stained by Levaditi's method. The organisms appear as irregularly twisted spirals, lacking the tight corkscrew appearance of the *Treponema pallidum*. (Smith, L. W., and Gault, E. S., 1942, Essentials of Pathology, ed. 2, New York, Appleton, Fig. 172.)

turns are regular and fairly angular, the depth of the spirals being 0.5 to 1  $\mu$ , and the distance between them 1  $\mu$ . The organisms are actively motile, rotating on their long axis at a fairly constant speed. Their trans-

lational movement is comparatively slow, but is usually sustained. Although the spirals ordinarily seem fixed, the organisms are extraordinarily elastic, and may at times elongate until the spirals almost disappear, and then snap together to assume their normal configuration. They may bend until the two ends meet to form a perfect circle, and remain in that position for many minutes, actively rotating all the time. This flexibility and elasticity, and the sustained uniform tempo of the rotation, help distinguish the organism from some of the saprophytic organisms often present in superficial lesions and with which it might be confused.

*T. pallidum* is difficult to stain with the ordinary aniline dyes, but reduces silver nitrate to form a black surface deposit which permits its demonstration in tissues, as in the Levaditi or Fontana stains. In electron microscope photographs (Morton and Anderson, 1942; Mudd et al., 1943) the spirals are more shallow and less well-defined than in the living treponemes, the organisms are thinner than they appear by either dark-field examination or after silver staining, and there is considerable shredding of the surface layers. There is evidence of a thin perioplastlike structure which encases the entire organism, and of multiple, fine, hairlike processes 14 to 17  $m\mu$  in diameter (flagellae?) not visible under the dark-field or in stained preparations. In these photographs one also sees minute, knoblike excrescences (150 to 500  $m\mu$  in diameter) attached to the side of the treponeme, and apparently an integral part of it, as well as knoblike structures within the organism approximately 50 to 100  $m\mu$  in diameter, and more dense than the surrounding protoplasm. Their significance, and their possible relationship to the much larger free globular bodies which are regularly seen in old cultures of saprophytic treponemata (cf. following section) are not yet clear. It is pertinent to note that in the case of *Borrelia recurrentis*, the shredding of the fine membranous perioplast and the appearance

of dense granules in the electron microscope photographs were observed only after centrifugation, and were not apparent in films prepared from electrodyalyzed suspensions not subjected to drastic manipulations (Lofgren and Soule, 1945). No differences have been observed in electron microscope photographs of pathogenic *T. pallidum* and of the nonpathogenic cultivable organisms purporting to be *T. pallidum*.

Although it has been suggested that *T. pallidum* may have a cyclic development, in one phase of which it is present in a granular and microscopically invisible form, this has not been proved. Failure to demonstrate the organisms in infectious tissue is of no significance, since one treponeme may suffice to produce the experimental infection (page 531). It has not been shown that the organisms can pass through the ordinary bacteria-tight filter.

#### THE PURPORTED CULTIVATION OF *T. PALLIDUM*

Despite numerous reports of the cultivation of *T. pallidum* on artificial media, there is good reason to believe that this organism has never been cultivated, and that the cultured strains represent in reality saprophytic contaminants which happened to be present in the syphilitic lesions. The conclusion is based on the following facts:

(1) Relatively few primary isolations from lesions have been reported, and other workers, using the same media have failed in their attempts to repeat these isolations (Kast and Kolmer, 1929).

(2) Once cultivated, the organisms purporting to be *T. pallidum* grow luxuriantly on relatively simple media, and differ materially from the organisms as seen in lesions. They tend to be polymorphic, varying in length, thickness and number of spirals. On the whole, they lack the delicacy of those seen in lesions, and do not give the same impression of elasticity; the spirals are not as regular or as fine, and motility

is not as uniform. The cultures often contain large numbers of semi-transparent globular bodies approximately 2 to 5  $\mu$  in diameter, with a reticulated and granular structure, and which may be adherent to spiral organisms. Whether they represent dead or degenerate treponemata, or whether they are forms which the treponemes can assume under certain environmental conditions, remains to be determined.

(3) Serologically, the several cultured strains purporting to be *T. pallidum* show wide differences, and some are serologically indistinguishable from known saprophytes. The only indication of a serologic relationship between the cultured and the pathogenic organisms has been the demonstration by Gaetgens that suspensions of some, but not all, of the cultured strains give positive complement-fixation tests with the serum of syphilitic patients. The organisms are, however, not specifically agglutinated, and other strains give variable to negative results (Beck, 1939; Eagle and Hogan, 1940; Koch, 1939-1940). Further, absorption of the serum of syphilitic patients with washed suspensions of, e.g., the reactive Reiter strain does not affect the Wassermann or flocculation reactivity of the serum with tissue extracts (Kolmer, Kast and Lynch, 1941; Eagle and Germuth, 1947).

(4) Despite occasional previous reports of pathogenicity, none of the cultured strains now available cause syphilitic infection.

Surveying the evidence, one must conclude either that these cultured strains never were *T. pallidum*, and represent instead saprophytic contaminants which happened to be present in the particular lesion, or, if they ever were *T. pallidum*, have so completely lost their pathogenicity and have so changed both morphologically and serologically that they are now in effect different organisms.

Attempts to grow the organism in fertile eggs or in tissue culture have hitherto been unsuccessful. The latter failure may in part



be due to the fact that the tissue cultures are of necessity aerobic, while the cultivable treponemata are obligatory anaerobes. In the infected rabbit, the organisms grow slowly. The incubation period of the disease varies with the size of the inoculum, with an average increment of approximately 4 days for each 10-fold decrease in the number of organisms inoculated (Magnuson, Eagle and Fleischman, 1947). It is a reasonable surmise that this difference in incubation period reflects the time required for the smaller inoculum to grow out. Although the many complicating factors make the calculation of dubious quantitative significance, this would imply a division time of approximately 30 hours, an estimate confirmed by the direct enumeration of organisms in developing rabbit chancres (Cumberland and Turner, 1948). The division time in vitro of the cultivated, nonpathogenic Reiter strain averages 10 hours.

#### RESISTANCE TO PHYSICAL AND CHEMICAL AGENTS

Suspensions of *T. pallidum*, obtained from acute syphilomata in rabbit testes, remain motile in vitro for a period of from 6 to 24 hours, and longer at icebox temperatures than at 25° or 37° C. Anaerobiosis favors their survival, as does the presence of serum and of tissue extractives. The organisms die on desiccation, even when lyophilized directly from the frozen state. When frozen at -80° C., a significant proportion of them remain motile and infectious for years (Turner and Fleming, 1939). In plasma, whole blood or serum stored at refrigerator temperatures the organisms remain viable for 24 hours, but not for 48 (Ravich and Chambers, 1942), a fact of importance in relation to the problem of transfusion syphilis. In the tissues, after death, they may remain infectious for 1 to 5 days. In infected animals, *T. pallidum* may apparently be killed by elevating the body temperature to 41.5° to 42°, and the treponemicidal ac-

tion of both mapharsen and penicillin in vivo is enhanced at those higher temperatures. Whether the therapeutic action of malaria in cases of neurosyphilis is due solely to the treponemicidal action of the higher temperatures, whether the body's natural defense mechanisms are enhanced at those higher temperatures, and whether antibodies are elaborated in the course of malarial infection which cross-react with *T. pallidum*, remain open questions.

The organisms are rapidly immobilized by trivalent arsenicals, bismuth and mercurials. There is reason to believe that these compounds are treponemicidal by virtue of their common affinity for —SH groups in the organism, and probably because they block essential —SH groups in enzyme proteins vital to the cellular economy. Like the immobilization of trypanosomes or the inactivation of certain enzymes, the toxic effects of arsenic, mercury and bismuth on *T. pallidum* are partially reversible. Under appropriate experimental conditions, organisms immobilized by these compounds may be reactivated by —SH-containing compounds such as cysteine, glutathione or 2,3-dimercaptopropanol. These remove the toxic metal from its reversible combination with the cellular constituents. Pentavalent arsenicals are inactive in vitro, which suggests that the therapeutic activity of these compounds is referable to their reduction to the trivalent compounds in vitro.

Although penicillin is therapeutically effective, the drug has no demonstrable direct effect on *T. pallidum* within the limited time over which such suspensions can be studied in vitro. This corresponds to the slow rate at which cultures of nonpathogenic treponemata (Reiter) are killed by penicillin in vitro. In vivo, penicillin in large doses causes the almost complete disappearance of spirochetes in syphilitic lesions within 9 hours (Tucker and Robinson, 1947; Turner, Cumberland and Li, 1947). The treponemicidal action of penicillin is enhanced both in vitro and in vivo by an

increase in temperature in the range from 37° to 42° C. (Eagle, Magnuson and Fleischman, 1947c).

#### HOST RANGE AND PATHOGENESIS

*T. pallidum* is the cause of syphilis, and in nature is confined to its human host. It has proved infectious in rabbits and monkeys, in which it causes infections which resemble the human disease in many respects, but which also show important differences. In mice and many other rodents the organisms multiply only slowly, without producing a significant tissue reaction, but are demonstrable by subinoculation into rabbits.

The human infection is usually transmitted by sexual contact. In men, although the organisms usually originate from lesions on the penis, they may originate anywhere in the genito-urinary tract, and be discharged with the seminal fluid. In women, organisms may derive from mucocutaneous perineal lesions, mucous patches on the vaginal wall, or from cervical lesions. In approximately 10 per cent of the cases, the infection may be extragenital, usually caused by a chancre or a mucous patch in the mouth, lip or tonsil. It is doubtful that the organisms can penetrate the intact skin; it is, however, possible that they can penetrate the thinner epidermal layer of the mucous membranes; and it is probable that in many persons they gain access through a break, perhaps only microscopic, in the epidermal layer. Although some of the organisms probably move away from the site of inoculation to reach the adjacent lymph nodes and thence cause a systemic infection within a period of hours to days, many apparently remain at the site of infection. When they have multiplied to a sufficient degree (or when the body's reactivity to their products has changed sufficiently) there is set up a characteristic inflammatory response known as the primary lesion or chancre, which

usually develops in 10 to 90 days. Two to 12 weeks thereafter, a generalized skin rash appears in most patients, with organisms demonstrable in the lesions. This secondary stage, which may also involve the mucous membranes, eyes, osseous system, and the central nervous system, reflects the generalized dissemination of the organisms and their multiplication at the foci; but the development of lesions may perhaps reflect the development of antibodies and an intensified response to the products of infection.

The subsequent course of the disease is extraordinarily varied and depends on the particular tissue involved. While approximately 25 per cent of the patients proceed to apparent spontaneous cure, and an equal proportion never again have symptoms referable to the disease, approximately half of those infected develop late complications, varying in severity and prognosis from the relatively benign gummata of the skin and bone to the prognostically serious cardiovascular or central nervous system involvement. In the late skin lesions the intensity of the cellular reaction is out of all proportion to the number of organisms demonstrable either by darkfield examination or by animal inoculation, strongly suggesting that the tissues have become sensitized to the products of the organisms.

In rabbits, the disease in its initial stages differs in no important respect from the disease in man. Animals may be infected via the eye, skin, testis or scrotum. One spirochete inoculated intratesticularly is regularly infectious, and 4 organisms inoculated intradermally cause infection in half the animals (Magnuson, Eagle and Fleischman, 1947). The time required for the development of a definite inflammatory lesion at the site of inoculation varies with the size of the inoculum, with an average linear decrement of 4 days for each 10-fold increase in the number of spirochetes inoculated in the range 1 to 100,000. This suggests a division time in vivo of approximately 30 hours. The slow rate at which the



organisms multiply in vivo, in both man and rabbit, is further indicated by the months which may elapse between the completion of inadequate treatment for early syphilis and the appearance of relapsing lesions. It is further evidenced by the fact that penicillin in aqueous solution can be administered once daily, and arsenicals as infrequently as once weekly, without prejudicing the outcome of treatment.

There are conflicting reports as to the speed with which the organism leaves the site of inoculation and invades the regional lymph nodes and the circulation, from as little as 5 minutes to as long as 4 to 24 hours. The rate of migration probably varies with the animal species, the particular tissue involved, its vascularity and lymphatic drainage, and the degree of tissue damage incidental to the inoculation.

The rabbit infection differs from that of man in several important respects. In man, a certain proportion of the cases apparently undergo spontaneous cure in a biologic sense, and the lymph nodes, even in untreated patients, remain infectious for animals in only a small number of instances (Lake and Bryant, 1930). The rabbit, however, remains infected for the rest of its natural life. Organisms persist in the lymph nodes, spleen and bone marrow, and can be demonstrated by inoculating those tissues into normal animals. Further, while some human cases of syphilis experience spontaneous cure, in others there are late complications which may affect almost any organ, and in which there may be extensive tissue involvement. In rabbits, on the other hand, once the early stages of the disease have healed, there is only a minor microscopic inflammatory reaction even in tissues known to harbor the organisms. Although the skin, bone and eyes may be involved in the early inflammatory process, late visceral manifestations (heart, liver and central nervous system) have only rarely been described. This difference in the degree of tissue involvement may be related to another im-

portant difference between syphilitic infection in rabbits and man. All infected rabbits can be apparently cured by either arsenicals or penicillin administered in adequate dosage. In men, however, mapharsen alone or penicillin alone will apparently cure only from 70 to 90 per cent of the cases, no matter how much drug is administered within the dosage ranges so far tested. Although this difference has not yet been fully explained it appears possible that, in the human infection, the organisms localize at foci not sufficiently accessible to either penicillin or mapharsen to permit their complete eradication.

Monkeys and chimpanzees are susceptible to inoculation with *T. pallidum* (Metchnikoff and Roux, 1903). The early course of the disease parallels that in man and in the rabbit, but it is not known whether the animals develop late complications resembling those seen in the human infection. Mice and rats were shown by Kolle and Schloszberger to harbor the organisms for long periods without developing gross lesions; and a variety of rodents have since been found to undergo a similar asymptomatic infection (Bessemans and de Moor, 1939; Wile and Sture, 1945).

#### IMMUNITY AND REINFECTION

Although reinfections with syphilis are not uncommon, they are usually observed in patients who were treated in the early stages of the disease, and only rarely in patients treated after the spontaneous disappearance of the secondary lesions. Two explanations are possible. The patient treated and cured months after the original infection may by that time have acquired a certain amount of immunity, sufficient to prevent reinfection. Alternatively, only early syphilis may be actually curable; thereafter, treatment may serve only to reduce the number of organisms, without wholly eradicating the infection. In such case, resistance

to reinfection would merely reflect the persistence of the original infection.

The extensive experimental studies of Chesney and his co-workers, confirmed by Uhlenhuth and Groszman and by Tani and his co-workers, have clearly demonstrated that, at least in rabbits, resistance to reinfection is not necessarily associated with the persistence of viable spirochetes. Animals adequately treated with arsenicals and apparently cured nevertheless resisted reinfection with a large number of organisms. Consistent with the clinical observations, this immunity was demonstrable only if the animal had been treated after a relatively long period, approximately 90 days after the original inoculation. Further, rabbits were more resistant to infection by the homologous strain than by heterologous strains (Chesney, Turner and Grauer, 1933). More recently, Magnuson has shown that resistance to reinfection is demonstrable as early as 6 weeks after the original inoculation, that it is first manifested by the suppression of the primary lesion and the development of an asymptomatic reinfection, and that the degree of immunity, measured by the number of organisms necessary to cause reinfection, increases progressively thereafter up to the longest time interval so far studied, 26 weeks. In animals treated and cured at that time, even 200,000 organisms fail to produce symptomatic reinfection, and there is complete immunity to smaller inocula.

The fact that there is a high degree of immunity in syphilis, sufficient to prevent infection with large numbers of treponemata, is of obvious significance with respect to the eventual possibility of mass immunization, but the mechanism of that immunity is not clear. Agglutinins, precipitins and complement-fixing antibodies have not been demonstrated in syphilitic serum, whether of man or rabbit. It is true that syphilitic infection does cause the appearance in the serum of antibodies capable of combining with a lipid present in mammalian tissue, and which give positive complement fixation

and flocculation tests with aqueous suspensions of that lipid. The probability that this is an antibody to a component of the treponema, and that the tissue lipid is serologically related to, but not necessarily identical with, that antigenic factor is discussed on pages 534 and 535. The relationship of this particular antibody to resistance to syphilis is, however, debatable. The antibody usually reaches its highest titer in early syphilis and before resistance to reinfection has become evident; conversely, in both animal and man there may be no demonstrable "Wassermann antibody" in the blood when a solid immunity has been established. It is possible that resistance to syphilis depends upon an antibody to a spirochetal antigen which is qualitatively distinct from that responsible for the development of positive Wassermann or flocculation tests, and which differs from the latter also in the time required for its elaboration.

There is, in fact, some evidence that the serum of syphilitic animals and men contains antibodies which may play a part in resistance to reinfection. Thus, if syphilitic serum is mixed with suspensions of viable *T. pallidum* and those mixtures are then inoculated into rabbits, the production of lesions is either delayed or prevented, despite the fact that there is no apparent morphologic change in the organism or reduction in its motility (Tani, 1936; Turner, 1936). Further, if syphilitic tissue is implanted into the skin of an immune rabbit, there is progressive death of the organisms in the implant, the spirochetolysis beginning in the periphery and progressing toward the center (Tani and Aikawa, 1940). In sections, no organisms can be seen after 21 days. In a repetition of these implantation experiments, Reynolds has shown that in immune animals the organisms are not demonstrable in the adjacent lymph nodes and that the implant loses its infectivity after 2 days. In normal animals, however, the organisms readily penetrate into the lymphatics to cause a generalized infection.



and remain viable in the implant for at least two weeks.

The possibility that there may be strain differences among *T. pallidum* is evidenced by the varying resistance of rabbits to reinfection with homologous and heterologous strains (Kolle and Schloszberger, 1926; Chesney et al., 1927, 1933).

#### DIAGNOSIS

The diagnosis of syphilis in its numerous manifestations rests on (1) clinical observation, (2) the demonstration of *T. pallidum*, usually by darkfield examination of the exudate from an open or abraded primary or secondary lesion, and (3) serologic changes in the blood and spinal fluid.

The diagnostic use of the darkfield examination is complicated by the fact that exudates from nonsyphilitic lesions may contain spiral organisms. Although most of these can readily be differentiated from *T. pallidum* by their larger size, coarser spirals and different type of motility, a few may be difficult to distinguish with certainty. On the other hand, a negative dark field does not necessarily exclude syphilitic infection. In the late stages of the disease, the number of spirochetes is extraordinarily small in relation to the degree of the inflammatory reaction, and the demonstration of treponemata, whether by staining, darkfield examination or even animal inoculation is rarely successful. Even in the early lesions, failure to detect the organisms in the exudate does not exclude their presence in large numbers. Thus, if a drop measuring 0.01 cc. is placed under a coverslip 22 mm. square and examined at a magnification of  $900\times$ , each field represents approximately  $10^{-6}$  cc. of fluid. The presence of 1 organism per field then implies the presence of  $10^6$  organisms per cc., but, conversely, the absence of visible treponemata in even a hundred microscopic fields is consistent with the presence of as many as 10,000 treponema per cc. of fluid. Fortunately, the number of

organisms in the exudate of early lesions is usually so large that one or two preparations suffice to detect them; when few in numbers, the organisms can usually be found by repeated darkfield examinations of the exudate over a period of several days, avoiding the use of local antiseptics.

The serologic diagnosis of syphilis is based on the demonstration in the serum (or spinal fluid) of an antibody to mammalian tissue lipids which combines with those lipids to give positive complement fixation, and flocculation tests (early literature summarized by Eagle, 1937).

Numerous theories have been adduced to explain this anomalous reactivity between syphilitic serum and tissue extractives. Sachs, Klopstock and Weil (1925) suggested that the antibody in question (termed "reagin" because of the doubt as to its proper identification) was a response to an antigenic stimulus provided by the host's own tissue lipids. Syphilitic infection presumably causes a breakdown in the host's own tissues, with the liberation of this lipoidal material. This, acting as a haptene, would be activated by the foreign protein of the treponeme to form a complete antigen, which would stimulate the formation of antibody both to the treponemal protein, and to the host's lipids. The fact that properly prepared extracts of many mammalian tissues and of some plants can be used as "antigen" in the diagnostic test would merely indicate that the reactive substance or serologically related substances are widely distributed in nature. Naturally, the simplest explanation for the anomalous reactivity of syphilitic serum with tissue extractives is that, as originally postulated by Wassermann, the antibody concerned is formed in response to *T. pallidum*, and not to the host's own lipids. One need only assume that one of the antigenic or haptenic components of the organism is serologically related to an ubiquitous tissue lipid. The latter thesis is strongly supported by the demonstration (Eagle, 1948) that

rabbits immunized with killed suspensions of pathogenic *T. pallidum* regularly develop positive Wassermann and flocculation tests.

The reactive material in the alcoholic tissue extracts used as antigen in these tests has been highly refined by Pangborn (1945). The purified lipid, termed cardiolipin, is alcohol soluble, acetone insoluble, contains 4 per cent phosphorus, but no nitrogen, and has an iodine number of approximately 120. Curiously, it is not fully reactive as such, but requires the addition of lecithin and cholesterol.

In practice, many complement-fixation and flocculation tests have been developed for the serum diagnosis of syphilis, all of which use as "antigen" suspensions of tissue lipids in an aqueous medium.

The flocculation tests all use as antigen an alcohol-soluble extractive of dried mammalian tissue (Davies, Eagle, Hinton, Kahn, Kline, Meinicke, Muller, Mazzini, etc.). Nonreactive impurities in the tissue are partially removed, usually by preliminary extraction with ether. "Sensitizing" or "fortifying" substances such as sterols, lecithin, or gums are added to the alcoholic tissue extractives. Dilution of this "fortified" alcoholic antigen with salt solution (the concentration and relative volume varying in the several tests) results in the formation of a milky suspension of lipoidal particles. The sensitizers apparently act by increasing the size or changing the shape of these particles, thereby, in an as yet unexplained manner, increasing their reactivity with syphilitic serum.

The minute particles of "antigen" disperse in normal serum, and this dispersion is the criterion of a negative result. In syphilitic serum, the particles combine with the antibody globulin ("reagin") to form the aggregates, microscopic or macroscopic, which are the criterion of a positive test. This aggregation, accentuated by shaking or by centrifugation, differs in no respect from, e.g., the agglutination of bacteria or the precipitation of dissolved protein by a

specific antigen. On the order of 0.003 to 0.01 milligrams of antibody protein per cc. suffices to give a positive flocculation test (Eagle, 1935; Davis et al., 1945); and a serum may contain up to 1,000 times that minimal reactive concentration. (Because the test is based on a cross reaction with a related antigen, and not with that primarily responsible for the formation of the antibody, the serum of syphilitic patients perhaps contains far larger amounts of antibody protein than this figure would imply.)

The Wassermann test chronologically preceded the flocculation procedures. The suspect serum is mixed with the finely dispersed tissue extractive which serves as "antigen," the aqueous suspension being more finely dispersed and more dilute than in the several flocculation tests. There is also added some fresh guinea-pig serum as complement. After storage at icebox temperatures for 4 to 24 hours (to permit the slow fixation of complement by the antigen-antibody compound formed if the serum were syphilitic), followed by a secondary incubation at 37° C. for ½ to 1 hour, a suspension of sensitized sheep red blood cells is added to indicate the presence or absence of residual free complement. If the serum had contained the suspected antibody, and if the complement had therefore been fixed by the lipid-antibody complex, no complement would remain free to act on the red blood cells. The opaque suspension of undissolved cells is the criterion of a positive complement-fixation test. If, in the absence of Wassermann antibodies, the complement had not been fixed, it would be free to act on the sensitized red blood cells. These, therefore, lyse to form a clear solution, which is the criterion of a negative Wassermann test.

With each Wassermann test there must be included an anticomplementary control of the serum, containing serum and complement, without "antigen," to make sure that the serum as such does not destroy complement. Obviously, if such is the case, the



Wassermann test (or indeed, any complement-fixation test) cannot be carried out on that particular specimen without special technics.

In both the Wassermann and flocculation tests, the degree of positivity may be expressed as the highest dilution of serum which gives a positive result. Alternatively, in the Wassermann test it may be expressed as the amount of complement which can be fixed under standard conditions by a given amount of serum.

**Spinal Fluid Tests.** Under normal circumstances, the antibody demonstrated in the serum of syphilitic patients does not pass into the spinal fluid, even in those with a high serum titer. In patients with central nervous system syphilis, the antibody is apparently elaborated locally and appears in the cerebrospinal fluid, sometimes in dilution titers exceeding that of the blood. The presence of a positive complement-fixation or flocculation test in the fluid is therefore usually indicative of involvement of the central nervous system. The Wassermann or flocculation titer of the fluid, coupled with its cell content and protein content, and their response to antisymphilitic treatment, provide information of diagnostic and prognostic value.

**False Positive Results.** Some sera, and less frequently spinal fluids, may give positive flocculation or Wassermann tests in the absence of actual syphilitic infection, and a number of conditions and infections have been described which, in a variable proportion of the cases, may cause the appearance of this cross-reactive antibody (B. D. Davis, 1944; Beerman, 1945). The development of technics to distinguish biologic false-positive tests from those due to actual syphilitic infection may greatly increase the reliability and utility of these tests (Neurath et al., 1947). Since the antibodies detected by the Wassermann and flocculation tests probably represent only one facet of the total antibody response to *T. pallidum*, and since positive tests may be induced by the injection

of the killed organisms, it appears probable that future progress in the development of serologic diagnostic methods may depend on the cultivation of *T. pallidum*, and on the production of a truly specific antigen, such as that originally envisioned by Wassermann.

#### TREATMENT

None of the methods or drugs so far developed for the treatment of syphilis can be considered wholly satisfactory. Prior to the discovery that penicillin was curative in syphilis, it had been shown that mapharsen (3-amino-4-hydroxy-phenylarsenoxide) administered 2 to 3 times weekly in individual doses of 1 mg./Kg. to a total of 24 or more injections would, if combined with weekly injections of bismuth preparations (e.g., 0.2 Gm. of bismuth subsalicylate suspended in oil) bring about the cure of more than 90 to 95 per cent of the patients treated. Unfortunately, approximately 1 in 120 of the patients treated 3 times weekly developed a severe toxic complication, and approximately 1 in 1,200 died (Eagle, 1944). Moreover, since treatment was of necessity given in outpatient clinics on an ambulatory basis, a large proportion of the patients failed to complete the scheduled 8 to 20 weeks of therapy and disappeared from observation after having received an amount of treatment sufficient to cause the healing of the local lesions, but not adequate to effect permanent cure, or even to prevent infectious relapse.

The picture was completely changed with the demonstration that penicillin can, in many cases, be curative within a period of 4 days to 2 weeks (Mahoney, Arnold and Harris, 1943). However, although penicillin is almost 100 per cent effective in the prevention of congenital syphilis, and although it is probably more effective than arsenic and bismuth in the treatment of neurosyphilis, it apparently effects cure in only 90 per cent of the cases of early infectious

syphilis. The remaining 10 per cent relapse either clinically or serologically in the following 18 months, whether treated with a total of 1, 2 or 4 million units, over a period of 4, 7 or 14 days. Whether the organisms in such relapsing cases are significantly resistant to penicillin, and the degree to which the possible appearance of relatively penicillin-fast strains may modify the therapeutic activity of the drug, remain to be determined. It is remarkable that an infection, which in the rabbit can be regularly cured by adequate doses of penicillin, is not uniformly cured in man. There is no evidence of a significant difference in the pharmacologic behaviour of the drug in the two species, nor of any difference in the susceptibility of the organism, which disappears from the lesions after treatment as quickly in man as it does in the rabbit. It is perhaps more than a coincidence that mapharsen alone also can produce cure in only 70 per cent of the human cases, although it also is regularly curative in rabbits. This suggests a difference in the pathogenesis of the infection in the two species. Thus, it is conceivable that in the human disease, organisms may in some of the cases reach the central nervous system, where the drugs do not penetrate in sufficient concentration for a sufficient period of time to kill all the organisms and effect cure. A similar situation has been observed in experimental mouse infections with *B. recurrentis*, where small doses of penicillin suffice to clear the blood, but enormous doses, as high as 200,000 to 400,000 units per Kg., are required to effect cure; it has been shown in that instance that resistance to treatment is associated with the presence of organisms in the brain (page 545).

#### EPIDEMIOLOGY AND CONTROL MEASURES

By and large, syphilis is acquired by sexual contact, extragenital infection accounting for only 10 per cent of the total. Primary and secondary syphilis, in which there are open lesions discharging millions

of organisms, are the most infectious stages, but patients may be infectious for months and occasionally for years after the secondary lesions have spontaneously disappeared. There is no evidence that different races or different individuals differ in their susceptibility or resistance to infection.

There are several obvious approaches to the epidemiologic control of syphilis. All have been used, with a particularly intensive effort in the past 10 years; and the degree to which the morbidity of the infection will be affected by these measures remains to be determined.

An important factor is the early and adequate treatment of the syphilitic patient in order to minimize the length of time during which he remains infectious, thereby reducing the number of persons to whom he may transmit the disease. Each case is potentially the source of a small outbreak; and not infrequently 10, 20 or even more cases may be traced, through several generations of infection, to a single individual.

The sources of infection should be traced to bring under treatment the infected individual from whom the patient acquired his disease, and who often is the focal point for many other actual and potential infections. The contacts to whom the patient may have transmitted the disease should also be found, and followed both clinically and serologically. Many will have clinical evidence of the disease by the time they can be brought in for examination. Their early and adequate treatment will obviously prevent them from transmitting the infection to others. Those contacts who are clinically and serologically normal at the time of the first examination may subsequently develop the disease, and should be kept under observation for several months. There is reason to believe that in such persons treatment with a relatively small amount of penicillin may effectively abort an infection not evident at the time of the first examination.

Calomel ointment applied to the genitalia after exposure has been used as a prophylaxis.



lactic measure for more than 40 years (Metchnikoff and Roux, 1903). In the experimental animal, the application of calomel in relatively large doses can prevent syphilitic infection because of the local effect of the calomel on the organism at the site of entry, and of its systemic effect on organisms which have migrated beyond the portal of entry into the adjacent lymph nodes and the general circulation. The value of this procedure is, however, debatable. In military personnel it has proved insufficient to reduce the incidence of venereal disease below a level of 30 to 40 per 1,000 per year, despite an intensive educational program. The striking increase in the incidence of venereal disease in the armed forces observed immediately after each of the last two world wars is further evidence for the inadequacy of such measures. Whether the infections occur because of failure to use the material provided, to use it properly, or to use it in time, or because local prophylaxis with soap and calomel ointment is in fact ineffective, is as yet unsettled.

In the experimental animal, phenylarsenoxides in propylene glycol or in soap solution applied to an open incision 1 to 4 hours after its inoculation with *T. pallidum* may prevent syphilitic infection by killing all the organisms before they can cause a systemic infection (Eagle, Magnuson and Fleischman, 1947a; cf. also Arnold and Mahoney, 1948). But even if calomel, arsenical or some other local prophylactic agent were effective when properly used, the fact that it must be applied within a few hours after exposure places a serious limitation on its practical utility. Further, and perhaps most important, it has been abundantly demonstrated that men thoroughly indoctrinated with the necessity and importance of mechanical or chemical prophylaxis usually fail to take advantage of facilities even when they are provided. In such cases, local prophylaxis apparently does not work for the simple reason that it is not used.

A new approach to the prevention of syphilis (and of gonorrhea as well) is afforded by the recent demonstration (Eagle, Magnuson and Fleischman, 1947b) that the amount of penicillin required to abort syphilitic infection varies with the size of the inoculum and in particular with the age of the infection. In animals inoculated with 2,000 organisms and treated within the first four days, the single abortive dose is a minute fraction of that necessary to cure the established disease. If the natural infection in man involves the penetration of the skin or mucous membranes by small numbers of organisms, then the infection may be susceptible to abortion by doses of penicillin so small (i.e., by effective blood levels maintained for so short a period) that even tablets taken by mouth may perhaps prove effective. Further, if *T. pallidum* multiplies as slowly in man as it apparently does in rabbits, then in contrast to chemicals applied locally, such peroral penicillin prophylaxis may prove effective even if given days after exposure rather than hours.

## TREPONEMA PERTENUE AND YAWS (SYNONYMS: yaws, pian, frambesia, etc.)

### HISTORY

*T. pertenue* was identified in the lesions of yaws by Castellani in 1905. Although it has not been cultivated on artificial media, its constant presence in the lesions, and its serial transfer through many generations in rabbits, with the regular production of characteristic lesions, have established its etiologic role. The disease has been recognized as a clinical entity for centuries, and may have been brought to the West Indies by infected African slaves. It is now worldwide in distribution and is endemic in the wet, hot climates of the tropical belt. In the cooler, mountainous areas, the disease runs a less florid course, the secondary lesions tending to localize in condylomatous form in the genitalia, perineum and axillae.

# BIOLOGIC PROPERTIES AND RELATIONSHIPS TO *T. PALLIDUM*

The organism is morphologically indistinguishable from *T. pallidum*, which it resembles further in its susceptibility to trivalent arsenicals and penicillin, in its viability for many years when kept frozen at  $-80^{\circ}\text{C}.$ , in its death on desiccation, in the fact that it has not yet been cultivated on artificial medium, in some of its serologic properties (the production of a positive Wassermann and flocculation test), in the histopathogenesis of the lesions, and the early progress of the disease. Further, there is evidence in both animals and man of a certain measure of cross immunity between the two diseases (page 541).

Whether the organisms are in fact identical, and whether yaws is actually syphilis which has been modified over the centuries by such factors as a hot climate, the varying human host, the earlier average age at which the disease is contracted, and the extragenital route of infection has been warmly debated for decades. As is indicated in Table 46, there are many points of similarity in the two infections, but there are also significant differences as discussed in the following paragraphs.

There appears to be no congenital yaws. Unlike syphilis, yaws is not transmitted to the fetus through the placenta. Tabes and paresis are rare, and the occasional reported case may be caused by unrecognized syphilis rather than yaws. Mucous patches, common in syphilitic infection, are uncommon in yaws. Although there are reports of central nervous system involvement and of aortic insufficiency occurring as complications of yaws, other observers have stressed the absence of visceral manifestations. The disease is not venereal, but is acquired by direct, extragenital contact, more than two-thirds of the cases being infected before the age of 15. The suggested role of the fly as vector requires further study.

In rabbits also, yaws differs demonstrably

from syphilis. Strains of *T. pallidum* isolated from human cases of syphilis in areas in which yaws is endemic cause rabbit infections which are indistinguishable from those caused by strains isolated in temperate climates. Further, these experimental infections with *T. pallidum* show consistent and significant differences from those produced by strains of *T. pertenue* isolated from cases in the same area (Turner and Chesney, 1934).

Whether *T. pertenue* and *T. pallidum* evolved one from the other, or whether they have always been biologically distinct organisms, they now cause recognizably different diseases in man; and the differences in the pathogenesis of the infections probably reflect fundamental biologic differences in the causative organisms. There is nevertheless considerable evidence of a close immunologic relationship between them (page 541); and a quantitative evaluation of the degree of immunity afforded by each infection against the other would be of interest and significance. The study of the antigenic structure of the two organisms and of their immunologic relationships must probably await their cultivation on artificial media.

## THE DISEASE IN ANIMALS AND MAN

In man, the "mother yaw" appears 3 to 4 weeks after exposure as a painless yellow-red papule ("framboesia" or strawberry) surrounded by an inflammatory zone. This gradually increases in size, erodes and ulcerates, the dried exudate forming a dark crust. Six weeks to 3 months later, sometimes after the mother yaw has completely healed, generalized secondary lesions develop which differ in no important respect from the primary lesion. When they localize in mucocutaneous junctions (mouth, nose, perineum) the lesions are moist and resemble syphilitic condylomata. Successive crops may appear over a period of several months to several years. The late sequelae of yaws are generally restricted to the skin and bone. Guma-



TABLE 46. SIMILARITIES AND DIFFERENCES BETWEEN THREE SYPHILISLIKE TREPONEM-  
ATOUS DISEASES OF MAN

	SYPHILIS	YAWS	PINTA
Causative organism....	<i>T. pallidum</i>	<i>T. pertenue</i>	<i>T. carateum</i> ( <i>T. herrejoni</i> )
Morphology.....	Morphologically indistinguishable		
Cultivation.....	Have not been cultivated on artificial media		
Epidemiology			
Mode of transmission	(1) Sexual predominantly (2) Transplacental to fetus	Person-to-person contact; non-venereal and not con- genital	Person-to-person contact; non-venereal and not con- genital
Age of infection.....	15 and upward, except for congenital infections	All ages, but two-thirds of cases before age of 15	All ages
Evolution of disease			
Primary lesions			
Site.....	Genitalia usually	Exposed areas, particularly of arms and legs	Exposed areas
Character.....	Indurated, ulcerated	Ulcerated	Non-ulcerated papule
Secondary lesions			
Site.....	Skin or mucous membranes	Skin or mucous membranes	Skin or mucocutaneous borders
Character.....	Maculopapular in skin; con- dylomata; mucous patches	Resemble primary lesion	Resemble primary lesion
Late manifestations	Any organ may be involved, with protean manifesta- tions: skin, bone, eyes, car- diovascular, central nerv- ous system, liver, etc.	Ulcerations of skin and de- structive lesions of bone; conflicting reports as to in- volvement of aorta, central nervous system or viscera	Depigmented and mottled areas of skin, hyperkera- toses
Reactivity in serologic tests with tissue ex- tracts (Wassermann and flocculation)			
Primary.....	+	+	+ (few)
Secondary.....	+	+	+ (60%)
Late.....	+	+	+ (100%)
Animals susceptible to inoculation	Rabbit, monkey Asymptomatic infection in mice and other rodents	Rabbit, monkey	?

tous nodules and deep, chronic ulcerations or crippling bone and joint lesions may develop. A destructive ulcerative mutilation of the rhinopharynx (gangosa), a proliferative exostosis of the upper maxilla (goundou), and juxta-articular nodules are also ascribed to yaws. Visceral complications are rare. Although aortic and central nervous system involvement have been described, most

workers agree that such complications are either rare or less frequent than in syphilitic infection.

In rabbits, intratesticular or intracuta-  
neous inoculation results in the appearance of a primary lesion, which develops more slowly, is less extensive and involutes sooner than does the corresponding syphi-  
litic lesions. There is also less tendency to

involve the adjacent tissues, although the histopathologic changes are basically similar in the two infections. Moreover, the organisms do not persist in lymph nodes after involution of the early lesions. In monkeys inoculated intradermally, primary lesions developed which resemble closely yaws as seen in man. Although generalized yaws are not usually observed, they may be produced by reinoculation. Nasal ulcerations and keratoses are also observed in these animals.

### IMMUNITY

In both animals and man, one attack of yaws may confer protection against a second attack. Immunity develops slowly; thus, reinoculation in man in the first 3 years of the infection may result in a modified attack; but most infected persons are refractory to reinfection after 10 years. There is some evidence that the late and crippling manifestations of yaws may be prevented if there is a sufficiently extensive and sufficiently prolonged secondary involvement. Among the West Indian slaves, it was common practice to auto-inoculate children who developed yaws without having a multiple secondary eruption, presumably in the belief that the late complications of the disease would be prevented if the early infection were generalized (Schöbl, quoted by Strong).

There is considerable evidence that yaws confers a measure of protection against syphilitic infection. Monkeys immunized to yaws by repeated inoculations over a period of 7 months were found to be immune also to infection with *T. pallidum* (Schöbl and Miyao, 1929). Four of 8 rabbits inoculated with yaws and treated 93 to 376 days later proved to be refractory to syphilitic infection; and in the other four the incubation period was prolonged and the lesions were small (Nichols, 1925). In man, almost all observers have commented on the relatively small number of cases of syphilis seen in

population groups heavily infected with yaws. In man as in animals, infection with yaws may therefore confer a measure of protection against syphilitic infection. However, protection is not absolute. Chambers (1937-1938) has reported 2 cases in which a child with congenital syphilis was born to a mother who had had yaws 20 and 2 years previously, and later cited an additional case in a woman who had had yaws for 10 years; while Findlay and Wilcox produced syphilitic infection by the subcutaneous inoculation of *T. pallidum* into a subject with a clear history of yaws 10 years previously.

There is some evidence also that syphilis may protect against yaws, or at least modify its clinical course. In syphilitic rabbits treated 17 to 63 days after inoculation, and reinoculated with *T. pertenue* 15 days later, Nichols noted a delayed development of the primary lesion, which was significantly smaller than those noted in normal animals. Syphilitic monkeys were resistant to yaws 11 months after the original infection, but not after 6 months (Schöbl, 1931). In man, cases of general paresis could not be infected with yaws (Jahnel and Lange, 1926; Strong, 1942), although the disease has been successfully transmitted to normal subjects by many workers.

### DIAGNOSIS

As in syphilis, the diagnosis of yaws depends on (1) the appearance of the lesions, (2) the demonstration of the organisms in early lesions either by direct dark-field examination, by contrast visualization with India ink, or by staining, and (3) the serologic test. Patients with yaws develop a positive Wassermann and flocculation test as regularly as do those with syphilis.

### EPIDEMIOLOGY

The disease is spread by direct contact with an open lesion discharging trepo-



nemes. The organism apparently cannot pass through the intact epithelium, and a cut or an abrasion, perhaps only microscopic, probably serves as a portal of entry. The role of flies as vector has been suggested by Kumm, who found that a fly (*Hippelates pallates*) widely distributed in the West Indies fed in large numbers on the open ulcerative lesions, and that *T. pertenue* could then be found in the foregut or stomach, where it remained viable for about 7 hours. The regurgitation of infective material on a breached area of the skin has been shown to cause infection in rabbits. *T. pertenue* has also been reported to pass through the intestinal canal of certain species of African flies and mosquitoes in viable form (Thompson and Lamborn, quoted by Strong). The early age at which infection occurs is a natural complement of the method of infection. Although man can be infected at any age, more than two-thirds of the infections occur before the age of puberty, and males are infected more commonly than females. The disease is usually considered to be restricted almost entirely to Negroes; however, Pardo-Castello has found the native white population in Cuba to be as commonly affected as the Negroes.

#### TREATMENT

The trivalent arsenicals, bismuth compounds and penicillin are effective against *T. pertenue*, and a small amount of treatment is sufficient to cause the disappearance of the lesions of yaws. Cases have not been followed with sufficient care to determine the minimal curative dose, or the frequency of relapse in relation to the dosage and schedule of treatment. There is, however, reason to believe that the dosage necessary to cure the infection, or at least to prevent subsequent clinical relapse, may be no less than is the case in syphilitic infection, and that a large proportion of cases receiving a small amount of treatment may subsequently relapse.

#### TREPONEMA CARATEUM AND PINTA

(SYNONYMS: mal del pinto, carate, azul, etc.)

Pinta is a disease characterized in its later stages by the presence of coalescing, depigmented and mottled areas on the wrists, hands, ankles, feet and scalp, and hyperkeratoses on the palms and soles. It is prevalent in Mexico and Colombia, is encountered in most of the American tropics and has recently been reported from the Philippines, Africa, India and the islands of the South Pacific. The number of cases in Central and South America is now estimated at approximately a million. Originally considered a fungus infection, its treponematal origin was indicated in 1938 by Saenz, Triana and Alfonso, who demonstrated the organism in exudates from lesions and in fluid expressed from the adjacent lymph nodes.

The organism is morphologically indistinguishable from *T. pallidum*. A confusing multiplicity of names has already been applied to it, among them, *T. carateum*, *T. herrejoni*, *T. pictor* and *T. pintae* (Perez-Vigueras, 1940). Although it has not yet been cultivated on artificial media, its regular presence in the lesions and the successful transfer of the disease from man to man (Leon y Blanco, 1939), with the recovery of the same organism from the induced lesions, seems to establish its causal relationship. A rabbit is said to have been inoculated successfully with organisms from a human lesion. Although four rabbits subinoculated from that animal did not develop a lesion, a human subject simultaneously inoculated did develop a typical darkfield positive primary lesion in 47 days (Leon y Blanco and Otéiza, 1945).

The relationship of *T. carateum* to *T. pallidum* and *T. pertenue*, its possible identity with one or the other of these two organisms, and their serologic interrelationships, are still speculative (Beerman, 1943). The

elucidation of these points may require the cultivation of these organisms on artificial media.

The disease may be contracted at any age and is first evidenced by a nonulcerating primary lesion. This is followed, in 5 to 18 months, by the appearance of successive crops of flat, erythematous and hyperpigmented lesions (pintid). After several years one observes the characteristic late depigmentation and hyperkeratoses. Although there were at first thought to be no late visceral complications, Saenz has reported cardiac involvement in 23 per cent and central nervous system involvement in 10 per cent of the cases.

The disease is restricted to Negroes and has to date not been serially transferred in animals. Human beings have been successfully inoculated with the exudates from the lesions, but reinfection does not succeed in the late cases. Syphilitic subjects have been successfully inoculated with pinta, and subjects with pinta may contract syphilis (Herrerón, 1940), indicating at least an immunologic differentiation between the two infections. Also unlike syphilis, the Wassermann test is not usually positive during the initial stage, becoming positive only during the secondary stage in about 60 per cent of the cases, while almost all of the late cases are seropositive.

Transmission is not venereal, but usually occurs by person to person contact. Flies (*Hippelates*) allowed to feed on serous fluid containing the treponemata have been shown to be capable of transmitting the disease to man (Blanco and Parra, 1941).

The disease responds to treatment with arsenicals and bismuth as do yaws and syphilis. There are as yet no reports as to the efficacy of penicillin.

#### TREPONEMA CUNICULI AND RABBIT SYPHILIS

*T. cuniculi* was identified by Bayon in 1913 as the cause of a natural venereal in-

fection of rabbits first described by Ross (1912). The organism is morphologically indistinguishable from *T. pallidum*, which it resembles also in its susceptibility to arsenicals. The natural lesion consists of superficial, scaly, eroded lesions on the genitalia and the adjacent perineal region. Inoculation into the skin of that area reproduces the disease; and intratesticular inoculation causes, in 14 to 28 days, an inflammatory reaction resembling that caused by syphilis but less extensive, lacking the induration characteristic of the latter disease, and consisting largely of fine nodules in the parietal layer of the tunica vaginalis, without marked enlargement of the testis (McLeod and Turner, 1946). In the following one to six months, metastatic lesions may be seen in the testis, scrotum, anus, prepuce and glans, and secondary lesions in the skin and mucocutaneous borders of the eyes, nose and mouth. Reactivity with tissue lipid (Wassermann and flocculation tests) develops in the same proportion and to the same degree as in rabbits infected with yaws or syphilis.

#### BORRELIA RECURRENTIS AND TROPICAL RELAPSING FEVER

##### HISTORY

In the eighteenth century relapsing fever was sometimes confused with typhus fever, but the two were clearly distinguished by Henderson in 1843. The spiral organism causing the disease [*B. recurrentis* (*obermeieri*)] was first seen in the peripheral blood of a human patient by Obermeier in 1868, and the disease was reproduced in man by the injection of infected blood in 1874. In 1904 Ross and Milne showed that so-called African tick fever was also caused by a spiral organism demonstrable in the blood, and indicated its probable identity with relapsing fever. Shortly thereafter, it was shown that, as had been suspected, the body louse could also act as a vector.



## MORPHOLOGY

*B. recurrentis* is a highly flexible spiral organism, varying in length from 8 to 30  $\mu$ , 0.3  $\mu$  in thickness, and with 5 to 10 irregular and loosely wound spirals which average 1 to 2  $\mu$  in depth and 3  $\mu$  in width (Fig. 1E). The organisms are actively motile, with both rotational and transitional movement. The latter is, however, not always progressive, and in blood preparations the organisms may move back and forth within the same microscopic field. Unlike *T. pallidum*, *B. recurrentis* takes the usual bacterial stains. Electron microscope photographs of suspensions freed of salts by electrodialysis (Lofgren and Soule, 1945a) did not differ materially from those of stained or dark-field preparations. The organisms showed uniform regular spirals, smooth regular walls, and had faint pointed tips, with an occasional heavy terminal filament. The protoplasm was uniformly stippled. However, repeated centrifugation in distilled water caused swelling, granulation and partial fragmentation, with the appearance of numerous flagellalike fibers extending from the body of the cells. Large dense granules were also seen attached to the organisms or floating free. There is a suggestive similarity between these artifacts and the characteristics of treponemata in electron microscope photographs (page 528).

## CULTIVATION AND BIOLOGIC PROPERTIES

The organism has been cultivated on media enriched with serum or blood. The addition of peptone is said to favor growth, and oxygen must be present. However, growth is irregular, and the strains cannot usually be transferred indefinitely on artificial media. Wolman and Wolman have found that the organisms remain viable in culture for more than a year, but that such cultures stored at 37° C. lose their pathogenicity for mice within a few weeks. They have described a periodicity in the number

of organisms present in the culture, and suggest that the relapsing nature of the disease may not be conditioned entirely by the immunologic response of the host, but at least in part may reflect an innate characteristic of the organism. The organisms grow well in developing chick embryos and in particular in the chorio-allantoic membrane, (Oag, 1940; Chen, 1941), despite the fact that newly hatched chicks are immune to infection and despite the fact also that chicken serum, even from the embryo, is bactericidal for the organisms. In the specific tick host the organisms survive for years and are carried through repeated generations by way of the ova.

In infected blood stored at -48° C., the organisms remain viable and infective for more than 27 months (Lofgren and Soule, 1945a), and the organisms in culture survive at from 0° to 12° C. for more than 8 months (Wolman and Wolman, 1945).

The widespread distribution of the disease and the several modes of transmission have led to the isolation of a large number of strains differentiated primarily on the basis of the area of isolation, or the vector concerned in their transmission, rather than by inherent biologic differences in the organisms. Although some strains will apparently grow preferentially in certain species of ticks, this may not reflect a genetic difference. Louse-borne strains have been shown to be infective for ticks, which can then transmit the disease to man; and naturally tick-borne strains have been similarly transferred to lice. Adler and Ashbel have also demonstrated the transmissibility of tick-borne relapsing fever (*S. persicus* in Palestine) to lice, but they question the epidemiologic importance of the "unnatural" vector.

Although agglutinins and other antibodies can be produced in high titer, the serologic differentiation of the various strains is complicated by the fact that their antigenic structure apparently changes repeatedly during a single infection. It has been

suggested that the antibodies first elaborated act as a selective factor which permits the survival only of antigenically distinct mutants, and that the characteristic relapsing course of the disease is due to the multiplication of these resistant mutants, for which the host must now elaborate new antibodies (Cunningham, 1925; Meleney, 1926, 1928).

#### PATHOGENESIS

The disease in man usually begins with an acute febrile onset 3 to 10 days after inoculation by the louse or tick. In this initial febrile stage there may be large numbers of organisms in the blood, they may be found in the urine in approximately a fourth of the cases, and by animal inoculation can sometimes be demonstrated also in the cerebrospinal fluid. After an average of 4 days the fever declines, coincident with the disappearance of organisms from the blood. As the number of organisms decreases, they become less motile, tend to assume bizarre forms and may agglutinate in rosettes. During the afebrile period, the blood is not infectious for lice. The afebrile period may last 3 to 10 days, and is followed by a second febrile attack during which organisms reappear in the blood, but in smaller number. The 3 to 10 such recurring febrile attacks give the disease its name. The mortality in the endemic infection varies between 2 and 5 per cent, but in epidemics it may be 50 per cent or even higher. In fatal cases organisms are found in sections of the spleen and liver, and are particularly numerous in the malpighian bodies of the spleen, which show miliary necrotic lesions. Hemorrhagic lesions may be found in the gastro-intestinal tract and in the kidney.

Monkeys, mice and rats can be inoculated subcutaneously, intravenously or intraperitoneally. Chinese hamsters (*Cricetulus griseus*) and guinea pigs are also susceptible, the organisms surviving in the brain of the latter for more than 3 years (Sergent,

1945). Monkeys show the characteristic relapsing course of the human disease. Organisms appear in the blood in 24 to 48 hours and disappear spontaneously in two to five days. In young rats (40 to 80 grams), they may appear in the blood in huge numbers within 24 to 72 hours, and disappear with extraordinary rapidity within a few hours. Even in these animals, however, the disease is rarely fatal. Consistent with the demonstration of the organisms in the cerebrospinal fluid and brain in human cases, there is some evidence that in rats, mice and guinea pigs the brain may serve as a reservoir of infection, after the apparent disappearance of organisms from the circulating blood (Heronimus, 1928; Anderson, 1946).

#### IMMUNITY

Serum agglutinins and bactericidal antibodies are readily demonstrable in both the experimental and the human disease, and the termination of the individual febrile attacks, as well as the eventual cure, may be related to their appearance. It is significant that the organisms which appear during a febrile relapse often differ in their serologic reactivity from those present in the immediately preceding attack (Cunningham, 1925-1926; Cunningham and Frazier, 1934). In squirrels inoculated with a single human strain, Meleney isolated 6 serologically distinct strains during a series of relapses. There is no fixed order in which the mutant strains appear in the course of a single infection, but there is an indication that the organisms in alternate relapses may be more closely related in their antigenic structure than is the intervening mutant. The development of mutant strains not susceptible to the antibody previously elaborated may therefore be the actual cause of the relapse, and final cure may reflect an increasingly broad immunity afforded by the multiple mutant strains. It must be noted that Ashbel has observed relapses without demonstrable changes in immunologic reactivity, and Stein



has furnished evidence of a common antigen in a number of supposedly different strains isolated from both animals and man.

Recovered patients have been found to resist reinfection 2 to 5 years later. Other workers, however, have found that patients remained immune only so long as the organisms persisted in the tissues. Attempts to immunize animals with killed borrelia have been unsuccessful; and it has not yet been possible to characterize and differentiate strains on the basis of their antigenic reactivity. There is evidence of cross protection between tick-borne and louse-borne strains. Monkeys infected with the tick-borne California strain proved resistant to reinfection with a louse-borne Chinese strain; and in hamsters, when the order of inoculation was reversed, the second infection consisted of a single short attack, with no relapses (Chen, Zia and Anderson, 1945).

#### DIAGNOSIS

The diagnosis rests primarily on the demonstration of the organism in the blood, either by direct dark-field observation, by the examination of stained blood films, or by animal inoculation. Young white rats weighing from 30 to 80 grams are particularly susceptible, organisms appearing in the blood in large numbers in 24 to 72 hours.

#### TREATMENT

Arsenical, bismuth preparations and penicillin have all been used. In adults, from 0.3 to 0.9 grams of neoarsphenamine or 40 to 60 milligrams of mapharsen is the usual dose. The therapeutic efficacy of the arsenicals is, however, open to question. Some observers (Taft and Pike, 1945) do not believe that they prevent relapses; while Wolman found that arsenical treatment reduced the number of true relapses and the interval between them without, however, affecting the mortality of the disease. Bismuth subsalicyl-

ate suspended in oil, and sodium potassium bismuth tartrate given intramuscularly in doses of 0.2 grams have been used in resistant cases.

The therapeutic efficacy of penicillin in human cases of relapsing fever has not been clearly defined. Penicillin given intramuscularly is curative in experimental animals, but large doses are necessary—of the order of 200,000 to 400,000 units per Kg.—perhaps because of the persistence in the brain of foci of infection (page 545), which are not reached by penicillin in sufficiently high concentration or for sufficiently long periods to eradicate them (Schuhardt et al., 1944 and 1946; Anderson, 1946). These findings are of interest in relation to the failures observed in the treatment of early syphilis with penicillin.

#### EPIDEMIOLOGY AND PREVENTION

In the endemic areas of Central and South Africa, over a wide area of Asia, and in the Americas, the disease is tick-borne. The most important vector is the genus *Ornithodoros*, many species of which have been shown to be infected in nature (Table 47). There is some evidence that rodents may serve as natural reservoirs of infection for the tick. Organisms are found in all parts of the infected tick, may persist for years, and are transmitted to the ova for many generations, their infectivity for mice remaining unchanged (G. E. Davis, 1943). Some tick species transmit infection to man through the coxal fluid, while others introduce the organisms directly by a bite. The tick-borne disease is not usually epidemic, and the seasonal incidence is probably related to the prevalence of the vector. Infected ticks (*O. turicata*, *O. hermsi*, *O. parkeri* and *O. talaje*) have been found over a wide area of the United States (Wyoming, Montana, Idaho, Texas, Kansas, California, Colorado, Arizona and Utah), and they have been found also in Florida, New Mexico

TABLE 47. GEOGRAPHIC DISTRIBUTION OF VARIOUS "SPECIES" OF *Borrelia* RESPONSIBLE FOR RELAPSING FEVER AND THEIR VECTORS

	"SPECIES" OF <i>Borrelia</i> *	MODE OF TRANSMISSION	
		LOUSE-BORNE ( <i>Pediculus humanus</i> )	TICK-BORNE ( <i>Ornithodoros</i> )
Europe	<i>recurrentis</i> <i>obermeieri</i> <i>hispanica</i>	++	<i>O. erraticus</i> ( <i>maroccanus</i> ) <i>O. verrucosus</i>
Africa	<i>duttoni</i> ( <i>crociduri</i> ) <i>kochi</i> <i>rusi</i> <i>berbera</i> <i>aegyptica</i> <i>marocana</i> <i>sogdiana</i>	+	<i>O. moubata</i> <i>O. erraticus</i> ( <i>maroccanus</i> )     <i>O. savignyi</i>
Middle East	<i>persica</i>		<i>O. papillipes</i> ( <i>tholozani</i> ) <i>O. asperus</i> <i>O. lahorensis</i>
India	<i>carteri</i>	+	{ <i>O. tholozani</i> <i>O. crossi</i> <i>O. lahorensis</i>
Russia	" <i>latyshevi</i> "		<i>O. verrucosus</i> <i>O. neerensis</i> <i>O. tartakovskyi</i> <i>O. tholozani</i>
North America	<i>novyi</i> <i>turicatae</i> <i>parkeri</i> <i>hermsi</i>		<i>O. turicata</i> <i>O. parkeri</i> <i>O. hermsi</i>
Central and South America	<i>venezuelense</i> <i>neotropicalis</i>		<i>O. venezuelensis</i> <i>O. talaje</i> ( <i>rudis</i> )

\* Validity of identification as separate species questionable.

and Oklahoma. Ground-squirrel and prairie-dog burrows are sometimes heavily infested with ticks with a high incidence of spirochetes, suggesting that these and other rodents may serve as natural reservoirs of infection.

In northern and western Africa, in Europe and in parts of Asia, the disease is spread primarily by the body louse, *Pediculus hu-*

*manus*, which becomes infectious 4 to 5 days after the ingestion of infected blood, and remains infectious for 2 to 3 weeks. The infection in lice is not transmitted to the second generation (Wolman and Wolman, 1945). The disease is apparently transmitted when lice are crushed near a bite or scratch which provides a portal of entry for the organism. It is the louse-borne infection



which may become epidemic under conditions which lower the host resistance and favor the rapid multiplication and wide dissemination of the insect vector. Thus, epidemics have generally occurred in malnourished, overcrowded populations with poor personal hygiene, and have often been incidental to famine and war. The seasonal incidence, with increased spread in cold weather, probably reflects the heavier louse infestation in thickly clothed persons crowding together for warmth.

Although the causative organisms cannot pass through the intact skin, they can penetrate the mucous membrane (e.g., conjunctiva), and transplacental infection may also occur. The possible role of the bedbug in transmission has been suggested.

The risk of infection may be reduced by personal cleanliness, and by avoiding contact with persons carrying lice or ticks. In epidemics, the isolation of patients, large-scale treatment and the wholesale delousing of the population, as with DDT, are effective procedures.

#### SPIRAL ORGANISMS OF THE MOUTH, MUCOUS MEMBRANES AND MUCOCUTANEOUS BORDERS

The classification and differentiation of the numerous spiral organisms which have been cultivated from the mouth and genitalia is still a puzzling and unsolved problem. Some are now classed as borrelia and others as treponemata. Wichelhausen and Wichelhausen list 30 spiral organisms cultivated from the mouth alone in the years 1906 to 1938, and emphasize the inadequacy of the present methods of classification. Morphologic differentiation on the basis of size, the number of spirals, or motility is clearly of little significance, and particularly in view of the wide morphologic variations observed with a single strain under varying conditions of growth, and even under the same conditions of growth with different lots of media or serum. Hampp

has emphasized the unreliability of stained preparations in studying the morphology of these organisms, while classification on the basis of metabolic activities (e.g., production of  $H_2S$ , reduction of indicators, proteolysis) is, with the present meager and incomplete information, unsatisfactory.

It is probable that in the past different names have been assigned to the same organism, and that the present multiplicity of strains may, at least in part, reflect the inadequacy of their characterization. There is the further possibility that organisms morphologically identical, and classed as *T. microdentium*, for example, may actually be different strains. As already mentioned, it is likely that the organisms described as *T. pallidum* and presumably cultivated from syphilitic lesions are in reality saprophytic contaminants occasionally present in the lesions. One of these (the so-called Reiter strain of *T. pallidum*) has been shown by cross absorption of antisera to be serologically identical with the S-26 strain of mouth spirochetes isolated by Wichelhausen, and another (the Kazan strain), although not identical, contains a related antigen (Eagle and Germuth, 1947; cf. also Beck, 1939).

The majority of the spiral organisms isolated from oral or genital lesions grow on meat (heart) infusion broth enriched with 5 to 20 per cent serum, without addition of tissue, although the addition of fresh blood is said to favor growth with some strains. Most of the organisms are obligatory anaerobes. The nonpathogenic Reiter strain grows on a medium consisting of glucose, several amino acids, vitamins, a sulfur-containing reducing substance, and serum albumin (although liver extract had to be added to permit continued propagation in culture) (Whiteley and Frazier, 1947). More recently, it has been shown that in an otherwise inadequate basal medium arginine, acetate and any one of a wide variety of  $-SH$  or  $-S-$  containing compounds are, at threshold concentrations of serum,

essential for the growth of this Reiter strain (Eagle and Steinman).

### BORRELIA VINCENTI

In a diverse group of infections (tropical ulcer; Vincent's angina or ulcerative stomatitis; pulmonary spirochetosis), one finds large numbers of a delicate, short (5 to 10  $\mu$ ), actively motile spiral organism with a variable number of shallow, irregular turns. It is usually present in association with a coarse, thick, Gram-negative rod with tapered ends, which is often banded or beaded in stained preparations (*Bacillus fusiformis*). There is no agreement as to whether these two organisms are the actual cause of the necrotic lesions, or only secondary invaders. Thus, the infection has been ascribed by some to a herpeslike virus (Black, 1942), and by others to a predisposing vitamin or other dietary deficiency, which would permit the invasion of the tissues by normally harmless organisms of the surface of the mucous membranes.

Treatment of the mouth lesions has been both local (neoursphenamine paste, bismuth paste, sodium perborate wash), and systemic (arsenicals, sulfanilamide derivatives). The number of therapeutic agents which have been advanced indicates that none is wholly satisfactory. More recently, penicillin injected intramuscularly or administered topically as a paste, mouth wash or tablet has been reported by a large number of workers to be highly effective.

## LEPTOSPIRA ICTEROHEMORRHAGIAE AND RELATED ORGANISMS

### HISTORY

Spirochetal fever (also termed "spirochetal jaundice," or "Weil's disease"), was first described in 1886 as a febrile disease associated with jaundice, characterized by involvement of the kidney and of the spleen. The causative organism was isolated in 1915

by Inada and his co-workers, who also demonstrated the role of rats as natural vectors, inoculated guinea pigs from infected rats, and demonstrated the presence of the organism in each stage of that cycle.

*L. icterohemorrhagiae* is characterized by its extraordinarily fine spirals, so closely wound and so short that they may be visible on darkfield examination only as a series

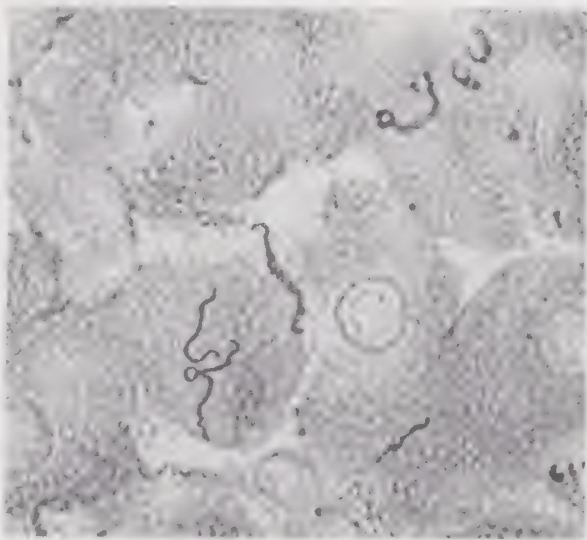


FIG. 30. *Leptospira icterohemorrhagiae* in the liver of a guinea pig inoculated with patient's blood. Levaditi's stain  $\times 885$ . (Photograph by Dr. W. Paul Havens, Jr., Jefferson Medical College, Philadelphia.)

of small dots, and are not usually distinguished in stained films. The length of the organisms may vary from 4 to 20  $\mu$ . It is approximately 0.1 to 0.2  $\mu$  in width, and moves by the active rotation of one end of the organism bent into a hook. Both ends may be motile, in which case the actively rotating organism has no translational motion. Electron microscope photographs show no evidence of flagellae, internal structure or granules (Morton and Anderson, 1943).

### MORPHOLOGY AND CULTIVATION

The organism grows best at 30° C. Various media have been described, most of which consist of an agar-meat infusion base



diluted with salt solution or water, and enriched with 2 to 5 per cent of defibrinated blood or serum. Gardner and Wylie recommend cultivation in 12 per cent rabbit serum diluted with glass-distilled water. Growth is obtained also on the chorio-allantoic membrane of the chick embryo (Morrow et al., 1938; Chabaud, 1939).

#### HOST RANGE AND PATHOGENESIS

A number of strains of leptospira have been isolated from human cases. The more important of these are *L. icterohemorrhagiae*, *canicola*, *hebdomadis*, *autumnalis*, and *grippotyphosa*. Their serologic relationships are not entirely clear, nor is it certain that they should be classified as distinct species. *L. icterohemorrhagiae* is the classic cause of Weil's disease. *L. canicola* was first isolated and differentiated serologically from *L. icterohemorrhagiae* by Klarenbeek and Schüffner in Holland, but similar strains have since been identified in the United States. It is not endemic in rats, has a wide distribution in nature in dogs, and has only a feeble virulence for guinea pigs. *L. grippotyphosa* is the cause of the disease known in Eastern Europe as "mud fever," "swamp fever" and "field fever," and for which field mice seem to be the natural vectors. *L. hebdomadis* and *L. autumnalis* have been isolated from cases in Japan, the former being the causative agent of a disease known as "seven-day fever." They are said to be serologically distinct from *L. icterohemorrhagiae* and from each other. From all of these strains, one may extract a serologically identical lipid which has limited complement-fixing activities with rabbit and human sera; there is said to be considerable overlapping in their antigenic structure.

Mice and rats are easily infected with *L. icterohemorrhagiae*. The rat is by far the most important vector in nature, remaining infectious for life. In young (3- to 5-week-old) Syrian hamsters, the organism causes infections fatal in 5 to 8 days.

Twenty-six species and subspecies of American rodents were found to be susceptible to infection, with death resulting in 3 to 13 days, usually preceded by jaundice (Packchianian, 1940).

*L. canicola* produces only a temporary bacteremia in young hamsters (Morton, 1942), the blood cultures remaining positive for 2 to 4 days. Randall and Cooper, however, were able to produce fatal infections in hamsters with this organism. *L. grippotyphosa* causes infections in rats which persist for life. In field mice, however, it causes a far more intense infection, with kidney involvement similar to that produced by *L. icterohemorrhagiae*, but rarely lasting for more than one month (Schüffner and Bohlander, 1943).

The disease in man has all degrees of severity, varying from infections so mild as hardly to call themselves to the attention of the patient, and recognizable only by serologic test, to serious, sometimes fatal, illnesses with deep jaundice and profound prostration. It usually begins after an incubation period of 6 to 15 days as an acute febrile illness which runs an irregular course. Conjunctival injection is a prominent and almost pathognomonic symptom. Although jaundice may appear in 2 to 3 days, at least 40 per cent of human cases do not exhibit visible jaundice (Meyer et al., 1939). Gardner and Wylie estimate that if one were to include the cases which are missed because of their mild nature and through the failure to do agglutination tests, fully 60 per cent of the total would be nonicteric. The central nervous system may be involved, with the appearance of the organism in the cerebrospinal fluid. The febrile illness subsides by lysis after 3 to 10 days, and is often followed by a second bout of fever. In fatal cases there are hemorrhagic lesions in the kidney, liver, skin, muscles or central nervous system. The mortality is extremely variable, ranging from 4 to 50 per cent in different out-

breaks, with an average of from 5 to 10 per cent.

#### DIAGNOSIS

The diagnosis rests on the demonstration of the organisms, and the appearance of specific antibodies in the serum. Early in the disease the leptospire may be found in the blood by darkfield examination of fresh specimens, or in thick dry films stained by the Giemsa technic.

Their presence can also be demonstrated by blood culture and by intraperitoneal inoculation into guinea pigs of the plasma obtained by light centrifugation. In positive cases, the organisms become demonstrable in the peritoneal cavity within a few days, or the characteristic pathologic picture will be found on the death of the animals in 10 to 12 days, with pleural and peritoneal hemorrhages. *Leptospira* are then demonstrable in many of the organs.

The urine may contain organisms, particularly from the tenth to twentieth day of the disease.

Antibodies may appear any time after the first week of infection. The agglutination titer rises slowly thereafter, and may not reach a peak for six to eight weeks, when it can be 1:30,000 or even higher. The titer falls slowly thereafter. Since agglutination is sometimes obscured by lysis at high concentrations of serum, it has been recommended that agglutination tests be performed with organisms preserved with 5 per cent formalin.

In evaluating the several diagnostic procedures in suspected rats, Larson found the order of decreasing sensitivity to be serum agglutination, Levaditi-stained sections, darkfield examination, animal inoculation, and culture, while Stavitsky (1945) states their reliability to be in the order of culture, darkfield, inoculation and stain.

#### IMMUNITY

The decrease in the number of organisms after the first 7 to 10 days probably reflects

the development of specific antibodies. The serum of convalescent patients protects guinea pigs and mice against an otherwise fatal inoculum of the organisms, and will cause lysis of the leptospire both in vitro and in vivo. Agglutinating antibodies have been found to persist in the blood of recovered patients for 1 to 20 years. Hyperimmune rabbit antiserum may have agglutination titers of 1:10,000,000; and 0.3 cubic centimeters of such serum administered even 72 hours after inoculation may cure the infection in white mice. At little as 0.003 cc. prevents infection if given prophylactically, at the time of inoculation (Larsen, 1943b).

#### TREATMENT

*L. icterohemorrhagiae* and *L. canicola* are sensitive in vitro to as little as 0.11 units of penicillin per cc. (Alston and Broom, 1944). Although penicillin treatment of the experimental disease is ineffective if given 6 to 7 days after inoculation, the proportion of survivors is increased with earlier treatment. Larson and Griffiths (1945) have recently found that penicillin and immune serum are equally effective in mice (*L. icterohemorrhagiae*) or hamsters (*L. canicola*) treated 17 to 48 hours after inoculation. Thereafter their efficacy decreased. Once symptoms had appeared, the majority of the animals could not be saved; and treatment was wholly ineffective when the infection had progressed to the stage of jaundice.

#### EPIDEMIOLOGY AND PREVENTIVE MEASURES

Rats are by far the most important vectors for *L. icterohemorrhagiae*, the organisms being found in their urine and feces. Many species are involved (e.g., *Mus norvegicus*, *rattus*, and *alexandrinus*); and the proportion of infected rats varies widely in different areas, from 4 to 55 per cent. Mice, voles (Japan), and bandicoots (India) may also serve as natural vectors. Human infec-



tion usually occurs from the ingestion of food, and especially of water, contaminated by the urine of infected rats. The organisms remain viable and infectious in water for as long as 22 days, and in moist soil for 3 months. The incidence of infection is highest in those groups whose occupation brings them into daily contact with water polluted by rats, e.g., miners, farmers, sewer workers and butchers. A significant number of infections also occur after bathing or accidental immersion in contaminated stagnant pools. The organism enters through breaks in the skin, or through the intact mucous membranes.

Dogs may also serve as natural reservoirs of infection, and may transmit either *Leptospira icterohemorrhagiae*, or *L. canicola* (page 550). Human cases traced to contact with infected dogs are usually due to the latter strain, and agglutinate it specifically.

The disease known as mud fever or field fever, and caused by *L. grippotyphosa*, is carried in nature by field mice (Uhlenhuth, 1943; Schüffner and Bohlander, 1943). The infection in these animals is an intense one which rarely lasts for more than one month. In consequence, mud fever is less endemic than Weil's disease, and appears in short summer or autumn epidemics. It is common in farm laborers, and flooding plays a large part in its spread.

Preventive measures include the extermination of the rat population which acts as the major vector and the prevention of the access of rats to water. Population groups whose work brings them in close contact with water contaminated by rats should protect the abrasions on the skin which serve as a portal of entry. This applies particularly to workers in water sewers and in mines and to those employed in fish industries.

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## 28

# The Bartonella Group

### INTRODUCTION

This group consists of *Bartonella bacilliformis*, the cause of Carrión's disease, and of micro-organisms of similar appearance, occurring in lower animals and classified in the genus *Haemobartonella*. These organisms infect erythrocytes and cause primary infectious febrile anemias. They are classified in Bergey's Manual in the family Bartonellaceae, order Rickettsiales.

### BARTONELLA BACILLIFORMIS

#### DEFINITION

*Bartonella bacilliformis* causes nonclinical asymptomatic infections and two very dif-

ferent and apparently unrelated conditions: Oroya fever, a febrile anemia of rapid evolution and high mortality, and Verruga peruana, a benign skin eruption. Each form has a distinctive pathologic substratum, and the two are linked immunologically. In Oroya fever, *Bartonella* is found on the erythrocytes. In both diseases it occurs within fixed tissue cells, notably those of the reticulo-endothelial system. On the red cells it is unmistakable and no other human parasite resembles it even slightly (Fig. 31). In the tissues it is intracellular and during Oroya fever multiplies within the cytoplasm

FIG. 31. All blood films were stained with Giemsa's fluid after fixation either by the May-Grünwald mixture or by methyl alcohol. (Weinman, D., 1944, Infectious anemias due to bartonella and related red cell parasites. Transactions of the American Philosophical Society, 33, 243-350.)

(1 and 2) *Bartonella bacilliformis* in blood films of two fatal cases of Oroya fever. In figure 1 the infection is intense; the bartonellae parasitize not only the erythrocytes but are also found within monocytes. (Original)

(2) The straight and curved rods, chains, dots, and rings illustrate the great morphological range of *B. bacilliformis* (Original, magnification as in 1)

(3) Section of an Oroya fever lymph node. Development of *B. bacilliformis* in distended endothelial cells lining the vein. The intracellular distribution in rounded clumps is distinct in the heavily infected cells. (Redrawn from C. Uribe)

(4) Section of a human verruga. Regaud fixation, Giemsa stain. The blood capillaries are numerous, and the proliferated endothelial cells conspicuous. *Bartonella bacilliformis* stains red, is very evident, and is often located distinctly within the cytoplasm of endothelial cells. Note that despite the numbers of parasites in the tissues none appear upon the erythrocytes. (Original)

(5) *Haemobartonella microti* in blood films of splenectomized mice. Variation in shape is extreme. Rods, coccoids, and rings occur both singly and in various combinations, constituting bow forms, filaments which contain rings or coccoids, rows of rings and coccoids, etc. (Redrawn from E. E. Tyzzer)

(6) *Eperythrozoon coccoides* in the blood of a splenectomized white mouse. An intense infection in which, as is customary, rings preponderate. The resemblance to certain forms of *B. bacilliformis* and *H. microti* is quite noticeable. (Original)

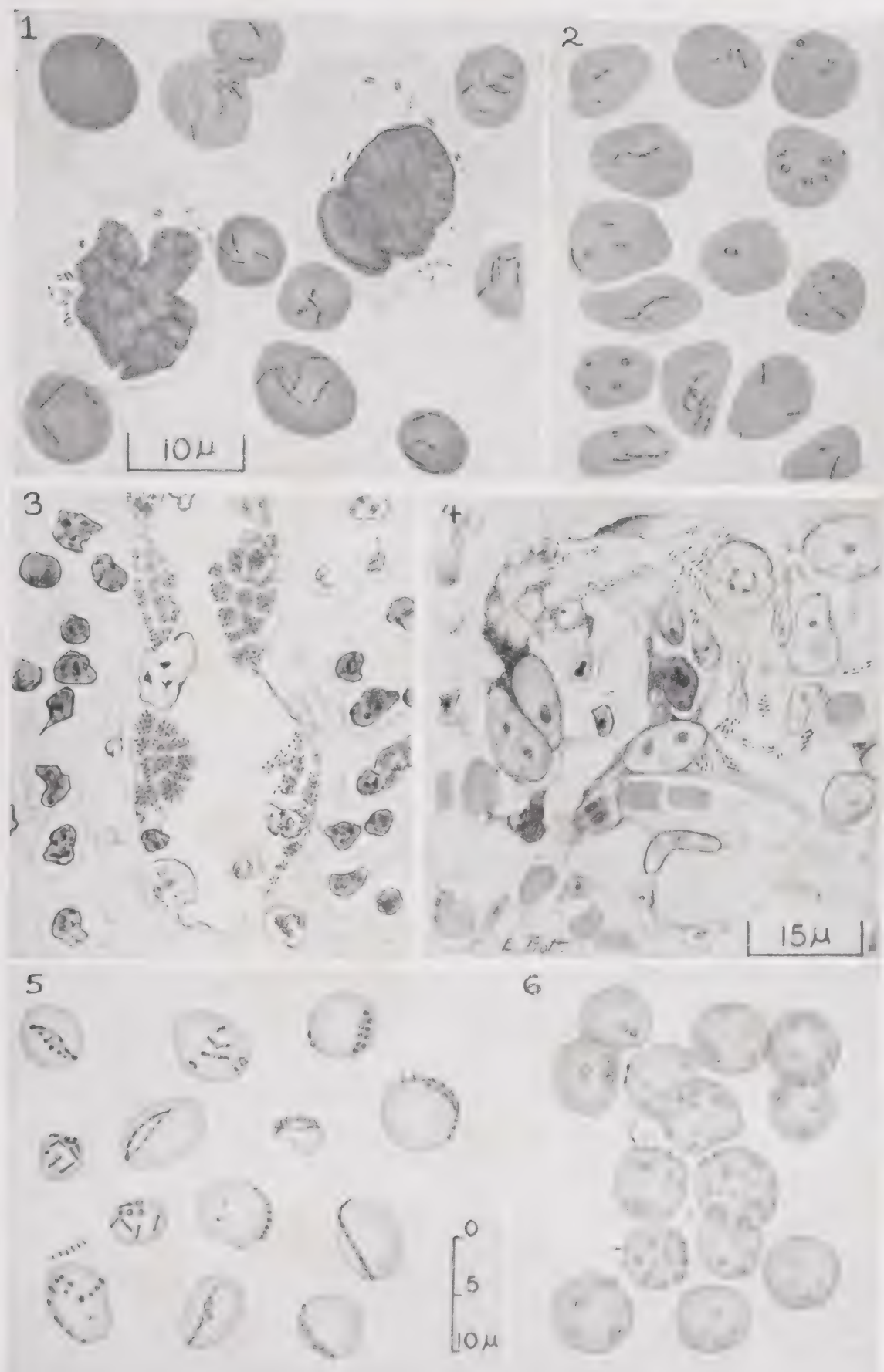


FIGURE 31



of vascular endothelial cells grouped in rounded masses or as isolated elements (Fig. 31). It can be cultivated by unlimited serial transfers on cell-free media.

#### HISTORY AND GENERAL CHARACTERISTICS OF BARTONELLOSIS

Bartonellosis is one of the most restricted of bacteriologic infections; it is exclusively American and exclusively tropical, being proved to be established only in Peru, Colombia and Ecuador. Verruga was known before the Columbian era, but the anemic form did not attract much attention until 1870 when an epidemic caused an estimated 7,000 deaths in the personnel constructing a railroad from the coast of Peru to the mountain mining city of Oroya. Credit for pointing out the bond between the two clinical forms goes to Carrión. Inoculated with verruga material, he died of Oroya fever 39 days later, thus strongly suggesting the etiologic unity of the two conditions. The micro-organism was reported by Barton in 1909 on the erythrocytes of Oroya fever patients. This was confirmed in 1915 by Strong, Tyzzer, Brues, Sellards and Gastiaturú, who also described the pathology of Oroya fever and discovered the pathognomonic lesion found therein. Noguchi and Battistini in 1926 cultivated the organism, described the relation of *Bartonella bacilliformis* to verruga and thus produced experimental evidence supporting the unitarian etiology. These results, not duplicated at first in other laboratories, were finally fully confirmed by the Harvard Expedition to Peru of 1937 (Weinman and Pinkerton, 1937; Pinkerton and Weinman, 1937); they also noted the prevalence of nonclinical or latent infections in man. Taken in conjunction with the discovery of the vector by Shannon and Noguchi, this fact has given a much clearer picture of the conditions for perpetuation and extension of the disease.

Oroya fever is a febrile macrocytic anemia

of rapid evolution and high mortality caused by *Bartonella bacilliformis*. The organism is readily visible in stained films; it becomes progressively more abundant in the blood with the aggravation of the anemia, and may infect over 90 per cent of the red cells. If the patient recovers from Oroya fever (the mortality rate of which is about 40 per cent), he usually develops the second form of the infection, verruga peruana. This is a benign condition with a death rate below 5 per cent and marked by a generalized eruption of very vascular, highly colored papules interspersed with more deep-seated nodular elements. Verruga persists, with successive crops, for a month to a year but, unlike Oroya fever, anemia if present is moderate in degree, and during all of this period bartonella will not be seen in blood films, although it can be cultivated from the blood.

#### MORPHOLOGY AND BIOLOGIC PROPERTIES OF BARTONELLA

*Bartonella bacilliformis* is a Gram-negative, flagellated organism which stains unsatisfactorily with the customary bacteriologic stains, but a distinct red violet with Wright's or Giemsa's fluid. It is very polymorphous, and the maximum morphologic range is seen in the blood of man (Fig. 31).

The culture medium most commonly used is a semisolid nutrient agar containing 10 per cent rabbit serum and a small amount of rabbit hemoglobin (0.50 per cent or less). In this medium *B. bacilliformis* remains pathogenic for several years at least, whereas blood agar slants are generally less favorable in this respect. Only media containing blood have been successful; serum or plasma has appeared essential, and hemoglobin even in small amounts is favorable, because it supplies hemin.

Growth becomes evident in a semisolid medium in a week to 10 days at 28° C., sometimes longer, in a band about 1 cm. wide and about 5 mm. below the surface.

Colonies, if minute, produce only a faint haze but they may grow to be visible white spherical bodies from 1 to 2 mm. in diameter. The organisms occur singly and in large and small irregular dense collections with jagged edges, 25 to 50  $\mu$  or more in diameter. Rod forms predominate in young (10- to 20-day) cultures and granules measuring as little as 0.2 x 0.3  $\mu$  in older ones. Flagella are demonstrable on motile strains by silver impregnation methods, they are unipolar and vary from 3.0 to 10  $\mu$  in length. *Bartonella* does not traverse the usual bacteriologic filters (e.g., Berkefeld N.).

The most favorable temperature, for the semisolid cultures, is 28° C. At 37° C. growth is just as abundant, but less prolonged. *Bartonella* is viable for months in citrated blood kept at refrigerator temperature ( $\pm 4^\circ$  C.) or in a semisolid medium at 28° C. The optimum pH is 7.8, with a range from 6.8 to 8.4. *Bartonella* is an obligate aerobe. It produces neither acid nor gas with any of the numerous carbohydrates tested, does not liquefy gelatin and has no action on lead acetate. All attempts to isolate a hemolysin have failed; it does not hemolyse red cells in culture or even localize on or about the erythrocytes when they are added to a semisolid medium. It is a facultative intracellular parasite when grown in tissue culture, and then reproduces the appearance seen in necropsy or biopsy material.

#### RANGE OF PATHOGENICITY

*Bartonella* manifests full pathogenicity for man only. In certain monkeys and apes, notably *Macaca mulatta* (*M. rhesus*), verrugas may be produced with great regularity and passed in series, but Oroya fever cannot be reproduced in animals at will. Usual laboratory animals, including the developing chick embryo, are not satisfactory, even for verruga production. The route of inoculation and the nature of the inoculum are of prime importance in determining the

character of the infection. Satisfactory inocula such as cultures, verrugas of either human or simian origin, or tissue from Oroya-fever patients, injected into the skin or subcutaneous tissue of *M. mulatta* produce a local verruga which is sometimes multiple, but very rarely generalized. Oroya-fever blood almost never produces verrugas in monkeys, yet cultures made from Oroya-fever blood will produce verrugas in almost every instance when appropriately injected. This is a puzzling feature and is not explicable by quantitative factors alone.

#### PATHOLOGY AND PATHOGENESIS

In Oroya fever the body is pale and somewhat icteric, the liver enlarged, the spleen frequently so and often infarcted, and the lymph nodes, usually enlarged, are often hemorrhagic. The microscopic appearance is determined by the development of masses of bartonella within the cytoplasm of cells lining the blood and lymph capillaries. The growth reaches an extreme degree, causes the cytoplasm to expand to many times its normal width and to bulge into the lumen of the vessel. The entire cytoplasm may be filled with micro-organisms which tend to form rounded masses or clumps. Infected cells are particularly abundant in lymph nodes and also occur in the liver, spleen, bone marrow, kidney, adrenals, pancreas, more rarely in the skin, heart and lung. Gross lesions such as the splenic infarcts may follow upon vascular occlusion by the swollen endothelium.

Verruga peruana differs as much in microscopic appearance from Oroya fever as do the clinical entities. It is a specific granuloma with three characteristic features. It is very vascular as a result of the formation within the element of numerous small-calibre blood vessels. Proliferated endothelial cells are present in abundance, they frequently occur in masses or islands and lie in edematous connective tissue, the whole being infiltrated with a variable number of



lymphocytes, plasma cells and polymorphonuclears. Lastly, bartonella is present in the lesion, usually within the cytoplasm of the tissue endothelial cells or in their neighborhood. Swollen vascular endothelial cells bulging into the lumen of the vessel, which is such a feature of Oroya fever, are almost totally lacking (Fig. 31).

The anemia of Oroya fever is due primarily to blood destruction, but the intimate mechanism of the hemolysis has resisted analysis. There is no evidence for the existence of a lysin of any sort in vivo, and *Bartonella* is not hemolytic in vitro. For verruga, there is at present no information correlating the complex cellular and tissue reactions with the properties or constituents of *Bartonella*.

The great variability in response to *Bartonella bacilliformis* infection, ranging from Oroya fever, to verruga not preceded by Oroya, and finally to nonclinical latent infection is probably due to variations in both the host and the micro-organism. Controlled experiments in animals are available with regard to verruga and asymptomatic infection, and do indicate a great range of host susceptibility and of pathogenicity of the organism. In Oroya fever both factors probably vary also, but since the condition is not reproducible at will in animals, the matter is not certain. No differences have been detected between bartonellae isolated from Oroya fever cases from those obtained from verruga patients. They are similar in pathogenicity, give various cross-immunity tests in animals, have the same morphology and behave similarly in vitro. Both flagellate and aflagellate strains are pathogenic.

#### IMMUNITY

The fundamental concept is that infection is long-continuing, and exhibits two successive clinical manifestations, of which the second, verruga, coincides with and seems to be an actual expression of immunity toward the first form, Oroya fever. If a pa-

tient has undergone Oroya fever, then has had verruga from which he has recovered, he will usually not have a second attack even though exposed. But should the second attack occur, it will almost invariably be verruga and not Oroya fever. This immunity to second attacks does not always signify that the infection has been eradicated. On the contrary, *Bartonella* can be cultivated from the blood of recovered patients for long periods of time. This latent or asymptomatic infection is a characteristic feature of the bartonelloses, animal as well as human. Latent infection is not limited to postconvalescence, and persons with no history of the disease can be carriers. Five to 10 per cent of the population in bartonella-infected areas are carriers and probably play an important part in maintaining endemicity.

Utilizing cultures as antigens, complement fixation has been reported. Agglutinins are present during both phases of the disease and in carriers. It is doubtful that they play any important part in acquired immunity. Limited attempts at immunization with formolized cultures induced agglutinin formation but did not prevent infection.

#### DIAGNOSIS

Oroya fever usually offers no diagnostic problem once the anemia is pronounced. Bartonellae can then be found in stained films by blood examination. Prior to the anemic period, blood cultures can be positive; the incentive to make them comes from knowledge of prior residence in an endemic zone and suggestive symptoms of the preanemic period: unexplained fever and joint and bone pains. A generalized, well-developed verruga eruption has a very distinctive appearance. Individual elements may resemble those of other eruptive conditions, but the histology is usually very different. Cultures from the verrugas are usually unsatisfactory, due to contamination, but cultures of *Bartonella* may be

obtained from the blood throughout the course of the eruption. The diagnosis of latent infection also depends upon blood cultures, which should be repeated, since not all may be successful.

The identification of *Bartonella bacilliformis* is based upon the following points: isolation from patients with a typical case of one of the two forms of the disease, or from carriers with a history of residence in endemic areas, or from infected sandflies; characteristic growth in a semisolid medium; pathogenicity for *Macaca mulatta*, in which verrugas may be produced; the morphology and cellular situation in vivo; and the appearance, staining and biochemical reactions in vitro. Some strains are non-pathogenic and for these, instead of the monkey test, tissue cultures can be substituted; growth will be both intracellular and extracellular, and within cells the organism will show a tendency to grow in rounded masses.

#### TREATMENT

It has been claimed that penicillin is active against *Bartonella*. Adequate doses of the drug may cause within 24 hours a reduction of 25 per cent in the number of parasitized cells and within a week it may no longer be possible to find any bartonella in blood films. The temperature frequently falls to normal within the first 48 hours; at about the same time or shortly thereafter the blood count becomes stabilized and then increases slowly. The minimum total dose is not yet established; to avoid relapses it is safer to give not less than a million units, distributed throughout 5 days to a week. The effect of the therapy may be followed by the count of parasitized cells. Doses of the magnitude stated may cure Oroya fever but do not eradicate the bartonella infection (Merino, 1945; Aldana and Muñoz, 1945). For the relief of the symptoms of the anemia, massive transfusions spread over long periods have been used success-

fully. These involve 3 to 8 liters or more and are given about 500 cc. at a time (Hodgson, 1947).

#### EPIDEMIOLOGY

*Bartonella* is transmitted by one or probably several species of *Phlebotomus* or sandfly indigenous to the endemic regions. There are records of a few cases of congenital infection, but bartonellosis is not transmitted by ordinary contact. The sources of the micro-organism are limited almost exclusively to the two hosts, man and phlebotomus, whence the importance of the asymptomatic infections. The 5 to 10 per cent of the apparently healthy population in endemic areas which have *Bartonella* circulating in the blood serve as possible sources of infection to phlebotomi.

Once implanted in a region, bartonellosis tends to remain almost indefinitely; in a focus reported in 1630, the infection is still endemic. Epidemics arise through infection of a previously nonexposed, and therefore nonimmune population. Such a population may be introduced within an endemic area to produce an "internal epidemic" not correlated with geographic extension (e.g., the 1870 Peruvian outbreak), or there may be an actual invasion of new territory. This seems to have been the case in Colombia in 1938, where bartonellosis caused an estimated 4,000 deaths, and now persists as an endemic infection.

Bartonellosis is contracted only in northwestern South America, exclusively in mountainous areas, solely in the neighborhood of river valleys where the elevation must be neither too high (below 8,000 feet) nor too low (above 2,500 feet), and only at night. The explanation lies in the biology of the vector. The pertinent facts are available for *P. verrucarum* in Peru. This phlebotomus is restricted to certain areas by requirements of moisture and temperature. Above a certain altitude the night temperature is too low; below a certain limit the rainfall is



insufficient and conditions are too arid. Transmission occurs only at night because the phlebotomi feed only then. Phlebotomi are the only natural vectors known, and *P. verrucarum* the only species for which transmission is proved. Very probably others are involved. In Colombia, where the Peruvian species have not yet been found, the epidemic was apparently not caused by the importation of infected sand flies but by the infection of native phlebotomi, possibly by human carriers.

#### CONTROL MEASURES

Since there is no vaccine of demonstrated efficacy and no therapy has yet proved to sterilize carriers, control measures have been directed against *Phlebotomus*, either directly or to hinder its access to man. Accurate information concerning the biology of the vector is available only for Peruvian species. In Peru an individual can protect himself by nocturnal removal from the endemic zone, or if nightly residence is required, by antiphlebotomus measures: sand-flyproofing of the sleeping quarters, use of repellents such as "6-2-2"\* or even oil of citronella, and the employment of DDT on and about the dwelling. These measures can be used on a small scale but, chiefly for economic reasons, have not been feasible for established communities in the Bartonella

region. The use of DDT may prove to be the simple, inexpensive measure needed for community sanitation, but although effective elsewhere (Hertig and Fisher, 1945), it is still in the trial phase in Peru.

#### HEMOBARTONELLA

The hemobartonellae resemble *Bartonella bacilliformis* in appearance, but do not cause any condition akin to verruga, do not multiply massively, if at all, within vascular endothelial cells, and are widely distributed geographically. They have a special interest because of the nature of the immunity associated with them. *Haemobartonella muris*, found in wild and many strains of laboratory white rats, provokes a fulminating anemia, accompanied by hemoglobinuria and death. A high percentage of rats are infected with *H. muris*, yet they show no signs of the disease, may never do so, and do not react to inoculation of the organism. But if the spleen be removed from these rats, the parasites multiply and the anemia evolves in its acute form. The infection is contracted at an early age and usually remains latent, probably throughout life unless splenectomy is performed. No hemobartonella infection is known to occur in man; the structures found on the erythrocytes after splenectomy in various anemic conditions have not been proved to be living organisms (Pappenheimer et al., 1945). The effect of splenectomy on *Bartonella bacilliformis* infections is not known.

\* A mixture of the repellents dimethyl phthalate, indalone and "Rutgers 612" (2-ethyl hexanediol 1-3) in the proportions 6:2:2.

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## 29

# Streptobacillus Moniliformis

### DEFINITION

*Streptobacillus moniliformis* is an aerobic, Gram-negative, pleomorphic micro-organism, which consists of irregular chains of bacilli interspersed with beaded swellings, and requires serum or commercial soluble starch for growth. It is a normal inhabitant of the nasopharynx of rats but is highly pathogenic for mice. In human beings it is the cause of one type of rat-bite fever as well as of a disease characterized by fever, rash and polyarthritis known as Haverhill fever or *erythema arthriticum epidemicum* which occurs without relation to rat bite. (SYNONYMS: *Streptothrix muris ratti*, *Haverhillia multiformis*, *Asterococcus muris*, *Actinomyces muris*, etc.)

### HISTORY

The micro-organism under discussion was reported by several authors from 1914 to 1918 as a *Streptothrix* (*Streptothrix muris ratti*, *Streptothrix teraxeri cepapi*, *Streptothrix longus* and *brevis*, *Streptothrix putorii*), which was recovered from human illnesses following the bite of rats, squirrels or weasels, and from the lungs of rats. In 1925, Levaditi et al. recovered it from the blood of a spontaneous human infection unassociated with rat bite and called it *Streptobacillus moniliformis*, the name which is now in common use. A disease, characterized by remittent cycles of fever, rash and arthritis, similar to that reported by Levaditi et al. (1925) and the syndrome follow-

ing rat bite, occurred as a milk-borne epidemic at Haverhill, Massachusetts, and became known as Haverhill fever or *erythema arthriticum epidemicum* (Place and Sutton, 1934). The micro-organism recovered by Parker and Hudson (1926) during this epidemic, and called *Haverhillia multiformis*, was subsequently also isolated from patients, exhibiting this syndrome following rat bite, and is generally agreed to be identical with *Streptobacillus moniliformis*. Further work showed that the organism is a normal inhabitant of the nasopharynx of rats, and that it is the cause of an epizootic disease in mice which, because of the localization of the lesions in the joints, heart, etc., had been erroneously compared with rheumatic fever. There is also ground for believing that the micro-organisms recovered in 1918 by Theobald Smith from the lungs of calves which had died of pneumonia and called *Bacillus actinoides* (subsequently classified as *Actinobacillus actinoides*), and similar ones isolated in later years from the lungs or middle ears of rats, also belong to the *Streptobacillus moniliformis* group. The great variety of names under which information relative to this micro-organism is scattered (reviewed by Brown and Nune-maker, 1942), has undoubtedly prevented its proper recognition as a cause of human disease; while only 8 cases of rat-bite fever due to this organism were reported in the



United States between 1916 and 1934, 31 cases due to it were recorded between 1939 and 1946 (Watkins, 1946).

Klieneberger's (1935) observation that all available strains of *Streptobacillus moniliformis* are associated with a pleuropneumonia-like component (L<sub>1</sub>) has been amply confirmed, but there is still disagreement as to whether it means (1) that *Str. moniliformis* actually represents an intimate symbiotic complex of two distinct micro-organisms (Klieneberger, 1942), or (2) that the L<sub>1</sub> component, while only a variant or special growth phase of *Streptobacillus*, is nevertheless a typical example of the pleuropneumonia group (Dienes, 1945), or (3) that the L<sub>1</sub> component bears only a superficial resemblance to, but cannot be regarded as a true member of, the pleuropneumonia group (Orskov, 1942).

#### CULTIVATION AND BIOLOGIC PROPERTIES

The addition of serum protein or egg yolk to the usual laboratory media is essential for growth, which occurs at 37° C. but not at 22° C. Heilman (1941) reported that commercial soluble starch (1 Gm. per 1,000 cc.) contained a growth factor for this micro-organism and could replace the protein in liquid media, but not in solid media; however, the L<sub>1</sub> component grew very sparsely or not at all in the starch medium devoid of protein. Dextrose starch or tryptose phosphate broth and agar, pH 7.6, are the basic media recommended by Brown and Nunemaker (1942). The final concentration of the agar should not be greater than 1.25 or 1.5 per cent. Horse serum, which is the best of the various animal proteins, and ascitic fluid with high protein content (over 3 Gm. per 100 cc.) stored in the filtered state, are added to the basic media to a concentration of 10 to 30 per cent directly before use. Growth is better under aerobic than under anaerobic conditions. Since high moisture content is necessary for optimum growth

on solid media, the Petri dishes should be sealed with parafilm or adhesive tape.

In fluid media, the organism usually grows in the form of fluff balls at the bottom and side of the tube, the supernatant liquid remaining clear and failing to reveal bacteria in stained smears. Growth is usually complete within 24 hours, and subculture may be difficult thereafter. The L<sub>1</sub> component grows more slowly and may still be viable after 8 days of incubation. On agar media, maximum growth is attained in 2 to 3 days, and the cultures remain viable for a week at 37° C. The colonies are raised and granular and measure 3 to 5 mm. in diameter. After 2 to 4 days of incubation, microscopic colonies of the L<sub>1</sub> component can be found buried in the agar beneath each larger colony; the streptobacillus surface growth can be washed away leaving the minute L colonies behind. After the *Streptobacillus* elements have died, an agar "cut-out" streaked across fresh medium can yield an abundant growth of L<sub>1</sub> colonies. Although these L<sub>1</sub> colonies can be maintained by serial subculture on solid media, it is usually possible to obtain growth of the streptobacillary component by culturing the seemingly pure L<sub>1</sub> colonies in fluid media. However, both Klieneberger (1942) and Heilman (1941) were able to obtain several L<sub>1</sub> strains which would not revert to or yield the streptobacillus. *Streptobacillus moniliformis* grows well in embryonated chicken eggs, but repeated attempts to infect the membranes of chick embryos with the L<sub>1</sub> component have been unsuccessful (Heilman, 1941).

Although the organism is visible in Gram-stained smears, morphologic details are better seen in preparations stained with Giemsa or Wayson's plague stain (Brown and Nunemaker, 1942). The morphology varies considerably with the age of the culture and the method of examination. Nevertheless, as the name implies, the basic components are streptobacilli varying from short rods to long, slender, interlacing fila-

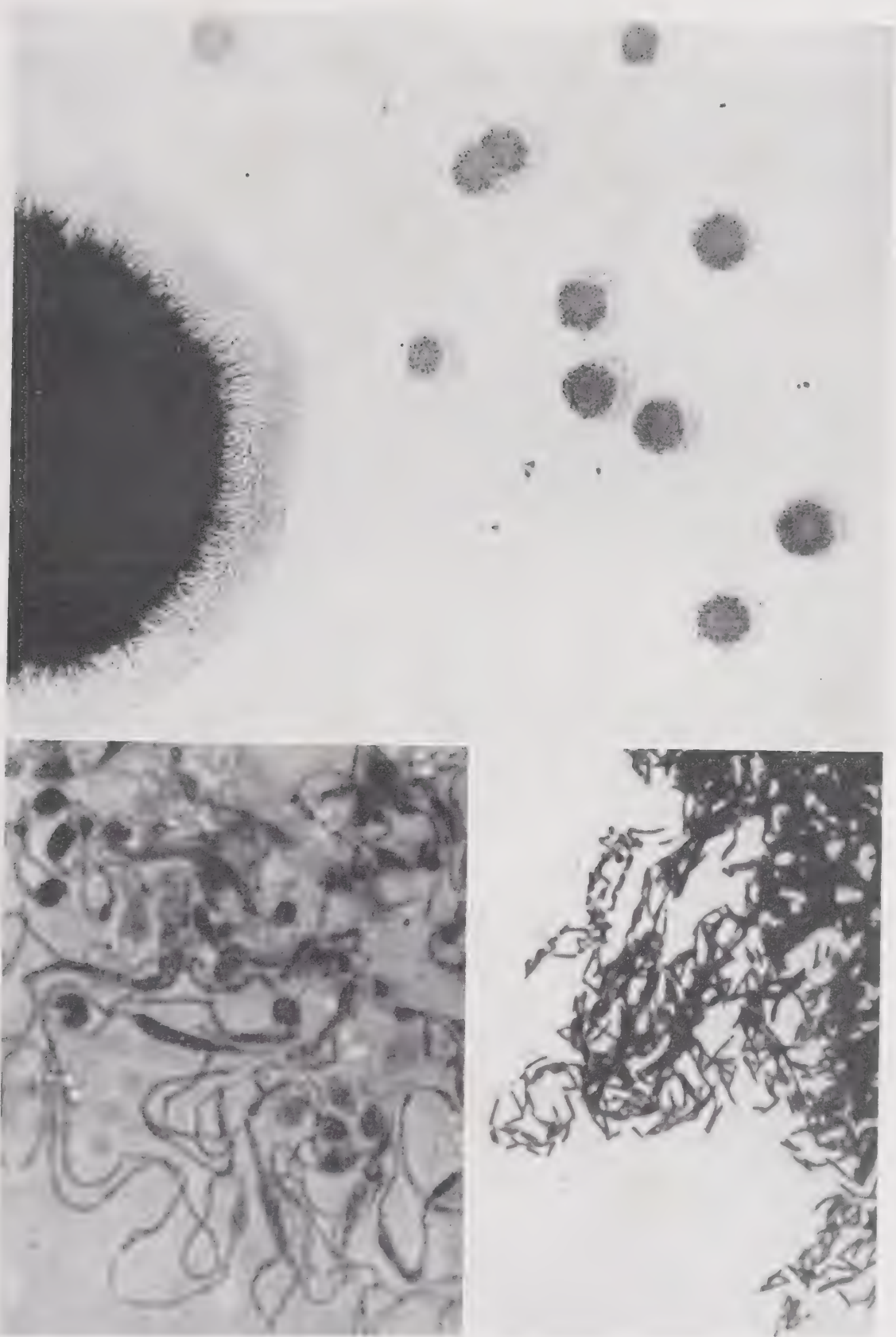


FIG. 32. (Top) *Streptobacillus moniliformis* and pleuropneumonia-like organism, L<sub>1</sub>. (24-hour culture on ascitic fluid agar stained in situ with methylene blue-Azure II.) Magnification,  $\times 112$ . (Dr. Cynthia H. Pierce.) (Bottom, left) *Streptobacillus moniliformis* colony. (24-hour agar culture stained in situ with methylene blue.) Note the irregular, wavy filaments containing deeply stained pear-shaped swellings. Magnification,  $\times 1855$ . (Bottom, right) Broth culture of *Streptobacillus moniliformis*. (Gram stain of 24-hour growth.) Note the lack of pleomorphism. Magnification,  $\times 1855$ . (Dr. Louis Dienes.)



ments (especially prominent in 24-hour cultures) which are irregularly fragmented into portions of unequal size (the dot-dash Morse code appearance). Along the course of these filaments, there occur large, round or spindle-shaped swellings, which, in older cultures, contain large numbers of granules. Opinion on the interpretation of the extraordinarily pleomorphic morphology of the *Str. moniliformis* is still divided between those who regard it as the resultant of the growth of two micro-organisms, i.e., the streptobacillus and the L<sub>1</sub> elements, and those who believe that all the different forms arise from a single reproductive unit. It is noteworthy that the basic components of the L<sub>1</sub> growth, whether on solid or liquid media, are large round bodies which contain granules.

Agglutination tests with sera prepared in rabbits indicate that the various strains of *Streptobacillus moniliformis* tested thus far are immunologically identical. The L<sub>1</sub> component has a distinct antigenic structure, and the complete streptobacillus complex contains antigens which are not present in L<sub>1</sub> (Klieneberger, 1942). It is of interest, furthermore, that the L<sub>1</sub> component has an antigenic relationship with some of the murine members of the pleuropneumonia group which are not associated with, or are a part of any known bacteria. However, human convalescent sera containing agglutinins for *Str. moniliformis* have failed to agglutinate any of the murine members of the pleuropneumonia group (Brown and Nunemaker, 1942).

#### DISTRIBUTION AND RANGE OF PATHOGENICITY

The natural habitat of *Str. moniliformis* is the respiratory tract of rats. It has been isolated in Europe, the British Isles, and the United States. It is transmitted to mice and human beings under natural conditions, and experimentally is also pathogenic for chick embryos. Sixteen to 20-hour cultures

injected intraperitoneally or intravenously in amounts of from 0.1 to 0.5 cc. produce a rapidly fatal infection in mice, terminating in the majority of animals in 24 to 48 hours. Prolonged cultivation (400 generations) in vitro may result in loss of virulence which cannot be restored by passage in mice. The L<sub>1</sub> component by itself is not pathogenic.

In human beings introduction of the organism into the skin (as after rat bite) or into the alimentary tract (as after consuming contaminated milk), results in invasion of the blood stream with subsequent localization in various parts of the body, and quite frequently in the joints. The special affinity for the joints is also seen in mice (Brown and Nunemaker, 1942) and chick embryos (Buddingh, 1944). In fatal cases ulcerative endocarditis or myocardial abscesses are prominent lesions.

#### DIAGNOSIS

Human infection with *Str. moniliformis* is characterized by a septic, occasionally relapsing, type of fever, morbilliform and petechial type of skin eruption, polyarthritides and leukocytosis. A syndrome presenting these manifestations should be suspected of being caused by this micro-organism not only when it follows (usually in less than 10 days) the bite of a rat or other rodent, but also when it occurs without reference to a bite. Clinical differentiation from rat-bite fever due to *Spirillum minus* (*Spirochaeta morsus muris*) may be difficult without help from the laboratory, although the occurrence of arthritis and the maculopapular and petechial type of rash is much more frequent in infection due to *Str. moniliformis*. Laboratory diagnostic methods include blood and joint-fluid cultures on the special media, mouse inoculation, and specific agglutination; the preparation of the antigen is described by Brown and Nunemaker (1942). An agglutinin titer of 1:80 or above is considered specific for in-

fection with this organism; the highest level may be expected between 1 and 3 months after infection, although specific agglutinins may disappear as early as 5 months after infection in some cases and persist in high titer for over 2 years in others. Skin tests with streptobacillary antigen have been found positive (a reaction of 1 to 3 cm.) 4 to 5 months after the infection.

### CHEMOTHERAPY

*Str. moniliformis* is susceptible to gold compounds (Brown and Nunemaker, 1942) and to penicillin (Heilman and Herrel, 1944; Altemeier et al., 1945), but not to sulfonamides and arsenicals. The administration of about 25,000 units of penicillin every 3 hours for at least 7 days is recommended for small children and proportionately larger dosage for adults (Altemeier, personal communication). It is noteworthy that penicillin is without effect on the members of the pleuropneumonia group.

### EPIDEMIOLOGY

The infection can be transmitted by the bite of a rat or other rodent (squirrel,

weasel) and by the ingestion of contaminated, raw milk (or its products). It is not known whether local infection of cattle by rat bite or systemic infection is responsible for the presence of the organism in the milk, or whether rats contaminate the milk directly. Eighty-six persons were affected in the only proved milk-borne epidemic on record which occurred in January, 1926, at Haverhill, Massachusetts (Place and Sutton, 1934). During May and June of 1925, there occurred at Chester, Pennsylvania, an outbreak involving an estimated 400 to 600 cases, which was at first diagnosed as dengue, but the absence of the mosquito vectors, the epidemiologic evidence of its relation to milk supply (unpublished report of Dr. Charles Armstrong to the United States Public Health Service, 1925), and the clinical similarity to the cases at Haverhill suggest *Str. moniliformis* as the etiologic agent (Place and Sutton, 1934). The mortality was nil in the milk-borne outbreak, but in the United States the case fatality rate of rat-bite fever has been 10 per cent. Sporadic cases without reference to any bites have also been recorded.

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## 30

# The Pleuropneumonia Group

### INTRODUCTION

The etiologic agent of bovine pleuropneumonia is the prototype of a distinct group of filterable, parasitic and saprophytic micro-organisms, which have not as yet been officially classified but possess properties that distinguish them from the ordinary bacteria, the filterable viruses, and the rickettsiae. The following criteria define the group: (1) growth in cell-free culture media with the development of varying types of polymorphic structures including large protoplasmic masses with "chromatin" bodies, globules, filaments, "rings" and, most important of all, filterable, elementary bodies, usually 125 to 250 m $\mu$  in size, which are the minimal reproductive units; and (2) the development on suitable solid media of minute colonies, as small as 10 to 20  $\mu$  and usually not larger than 600  $\mu$ , with a central, dark nipplelike structure or vacuolar mesh-work. Parasitic members of the group require protein for their growth, while the saprophytic members can grow in protein-free media (Sabin, 1941).

These organisms exhibit a high degree of host specificity. In cattle, one species variously known as *Pleuropneumonia bovis*, *Asterococcus mycoides*, *Coccobacillus mycoides peripneumoniae*, *Micromyces peripneumoniae bovis contagiosae*, *Mycoplasma peripneumoniae*, *Asteromyces peripneumoniae bovis*, etc., is the etiologic agent of the highly contagious disease known as pleuropneumonia bovis. In sheep and goats, another member of this group is the etiologic agent of a disease occurring in Europe and northern Africa known as agalactia, which affects males as well as females with particular involvement

of the joints and eyes, and in lactating animals also the mammary glands. A distinct species found in dogs, *Asterococcus canis*, has not been established as the cause of any naturally occurring disease. Micro-organisms of this group occurring in rats, sometimes also called "L organisms" (Klieneberger, 1940), have been proved to be the cause of a spontaneous "polyarthritis," but the etiologic relationship of the separate species recovered from lungs exhibiting bronchopneumonia and bronchiectasis has not yet been established. The many distinct species which have now been recovered from normal mice and shown to possess a variety of pathogenic properties under experimental conditions cannot as yet be correlated with any naturally occurring disease (Sabin, 1941). A pleuropneumonia-like micro-organism has recently been found in embryonated eggs (van Herick and Eaton, 1945), but it has not yet been definitely established that chickens are natural hosts or that it causes any disease in them. The natural occurrence of distinct species of this group in the genito-urinary tract of women, and much less frequently under normal conditions in that of men, has now been demonstrated beyond doubt (Dienes, 1940; Dienes and Smith, 1942, 1945; Klieneberger-Nobel, 1945; Beveridge et al., 1946; Salaman, 1946); however, their etiologic relationship to cervicitis, prostatitis, so-called "nonspecific urethritis" and Reiter's syndrome (urethritis, conjunctivitis and arthritis) is still being investigated.

### HISTORY

Bovine pleuropneumonia, a contagious disease of cattle, characterized by extensive

consolidation of the lungs, pleurisy, subpleural effusion and occasionally joint involvement, has been recognized in Europe for over 200 years, and is said to occur throughout the world with the exception of North America, Western Europe and India. The etiologic agent, for a long time regarded as a filterable virus, was first cultivated in a cell-free medium in 1898. Subsequent studies established the special type of colony and growth on solid media, the remarkable polymorphic character of the micro-organism, and the presence in cultures of "particles," 125 to 150  $m\mu$  in size, which are capable of reproducing not only themselves but also the larger and more complex structures of the growth cycle. The etiologic agent of agalactia of sheep and goats was cultured in cell-free media in 1923 and shown to differ only in pathogenic and immunologic properties from the organism of bovine pleuropneumonia. In 1934, similar organisms were found in dogs, and in 1935, Klieneberger (1935, 1942) first demonstrated that all available strains of the Gram-negative pleomorphic bacillus, occurring in rats and known as *Streptobacillus moniliformis* had a component, which she called  $L_1$ , bearing great resemblance to the pleuropneumonia group. Although the " $L_1$  organism" could be separated and maintained in pure culture without reversion to the parent *Streptobacillus moniliformis*, it was not recovered by itself from rats, with one possible exception; however, other pleuropneumonialike organisms, unassociated with bacteria and distinct from  $L_1$ , have been found in the lungs of rats. In 1936, Laidlaw and Elford recovered from raw sewage filterable micro-organisms which resembled the pleuropneumonia group in that minute particles, 125 to 175  $m\mu$  in size, reproduced the same types of polymorphic structures found in cultures of *Pleuropneumonia bovis* and *agalactia* and gave rise to the characteristic, minute colonies on solid media, but differed from them in not requiring protein for growth and in

being devoid of pathogenic properties. In 1938, immunologically distinct pleuropneumonialike micro-organisms were discovered in mice. Their special tissue affinities (Sabin, 1941), as well as certain similarities between the experimental conditions which they produce in mice with some of the manifestations of human rheumatoid arthritis and rheumatic fever, led to an extensive, ultimately unsuccessful, search for agents of this group in these human diseases. Organisms of the pleuropneumonia group have been found in the genital tract of women, and subsequently in the urethral and prostatic discharge of men, and their relationship to Reiter's syndrome and to certain human genito-urinary infections unassociated with ordinary bacteria have been investigated.

#### CULTIVATION AND BIOLOGIC PROPERTIES

Smears of animal tissues, exudates, or cultures in which a member of this group has multiplied, do not reveal any formed elements suggesting the presence of a micro-organism when stained by Gram method or by the ordinary aniline dyes suitable for bacteria. Staining by Giemsa or other methods suitable for protozoa yields the best results. In smears prepared from centrifuged sediments of fluid cultures, resuspended in small amounts of saline solution, the organisms, though faintly stained, are Gram negative. The morphology appears different according to the method of examination, i.e., stained films, dark field, annular oblique incident illumination (Klieneberger and Smiles, 1942) or agar fixation (Dienes, 1945). It varies with the conditions of growth, being different in colonies on solid media, in fluid cultures and in tissues, and with the species. The growth in fluid media, either stained with Giemsa or examined in the dark field, exhibits many different forms which may include rings, "triangles," "quadrangles," etc., with denser bodies distributed



irregularly through these structures, streaming filaments, spirillar and small bacilliform bodies, and elementary-body-like granules, depending on the type of micro-organism studied and the stage of growth (Fig. 33 J, K, L, R and S). Growth on solid media consists predominantly of large, plastic protoplasmic masses (easily distorted and disrupted on smear and probably also by fixation) which, apparently depending on the phase of growth, contain "chromatin" bodies (Q) of varying sizes and shapes, or large

numbers of granules or elementary corpuscles; in ordinary Giemsa-stained impression films it may appear as large disks, globules or even amorphous masses. While the development on solid media is of importance to the ultimate concept of the true mode or modes of reproduction, the morphology in fluid media is less confusing and more decisive for identification and differentiation of individual members of the pleuropneumonia group.

Suitable media consist of fresh heart

FIG. 33. The filterable micro-organisms of the pleuropneumonia group. Note how remarkably different are the forms seen in preparations from fluid cultures as compared with those found in impressions of colonies on solid media.

(A) "Fully developed colonies of pleuropneumonia (strain 'Shanghai') and others which have not yet acquired the clear peripheral rings.  $\times 70$ " (J. Path. Bact.).

(B) "Colonies of pleuropneumonia. Strain 'PP'.  $\times 70$ " (J. Path. Bact.).

(C)  $L_4$  colonies after 10 days' incubation.  $\times 80$  (J. Hyg.).

(D) "X" colonies of Sabin and Johnson (81) grown from human tonsils.  $\times 150$  (Proc. Soc. Exp. Biol. and Med.).

(E) Type-A micro-organism of mice. Colonies (3 days' incubation) photographed with transmitted light.  $\times 100$ .

(F) Type-B micro-organism of mice. Colonies (3 days' incubation) photographed with transmitted light.  $\times 100$ .

(G) Same as E, photographed with oblique lighting.  $\times 100$ .

(H) Same as F, photographed with oblique lighting.  $\times 100$ .

(J) Four-day culture of pleuropneumonia in serum broth, showing condensed rings and spherical elements. Giemsa.  $\times 1500$  (J. Path. Bact.).

(K and L) Five-day culture of pleuropneumonia in serum broth, showing predominance of "elementary bodies." Giemsa.  $\times 1200$  (J. Path. Bact.).

(M) Impression of 2-day-old pleuropneumonia colony on serum agar showing "moniliform elements" and "yeastlike" bodies. Giemsa.  $\times 2000$ . (J. Path. Bact.).

(N) Same. "Active peripheral growth with pseudopodial budding of the large yeastlike bodies. Giemsa.  $\times 2000$ " (J. Path. Bact.).

(P) Mesothelial cell of peritoneum of a mouse inoculated with brain tissue infected with a Type-A micro-organism, that had been maintained by animal passage and had not been cultured in vitro. Compare the typical forms of the micro-organism seen in the cytoplasm of the cell with those shown in R. Giemsa.  $\times 1600$ .

(Q) Impression of 6-day-old pleuropneumonia colony on serum agar. "Composite drawing of 'nucleated' bodies with chromatic elements surrounded by blue-stained sheath. Giemsa.  $\times 1500$ " (J. Path. Bact.). (The author has seen similar structures in cultures of the Type-B micro-organism of mice in the early passages after isolation from the animal body; they were present in fluid as well as solid media.)

(R) Two-day-old culture in serum-glucose broth of the Type-A micro-organism of mice. Note relatively simple morphology consisting of elementary bodies and rings exhibiting one or more dense bodies. Giemsa.  $\times 1000$ .

(S) Two-day-old culture in serum-glucose broth of the Type-B micro-organism of mice. Note the more complex morphology, especially the filaments "growing out" of the rings. Giemsa.  $\times 1000$  (J. Bact., Ref. 82).

(Sabin, A. B., 1941, *Bacteriological Reviews*, 5, 1-66.)

Figures A, B, J, K, L, M, N, Q are reproduced from Ledingham (53) and figure C from Klieneberger (48).

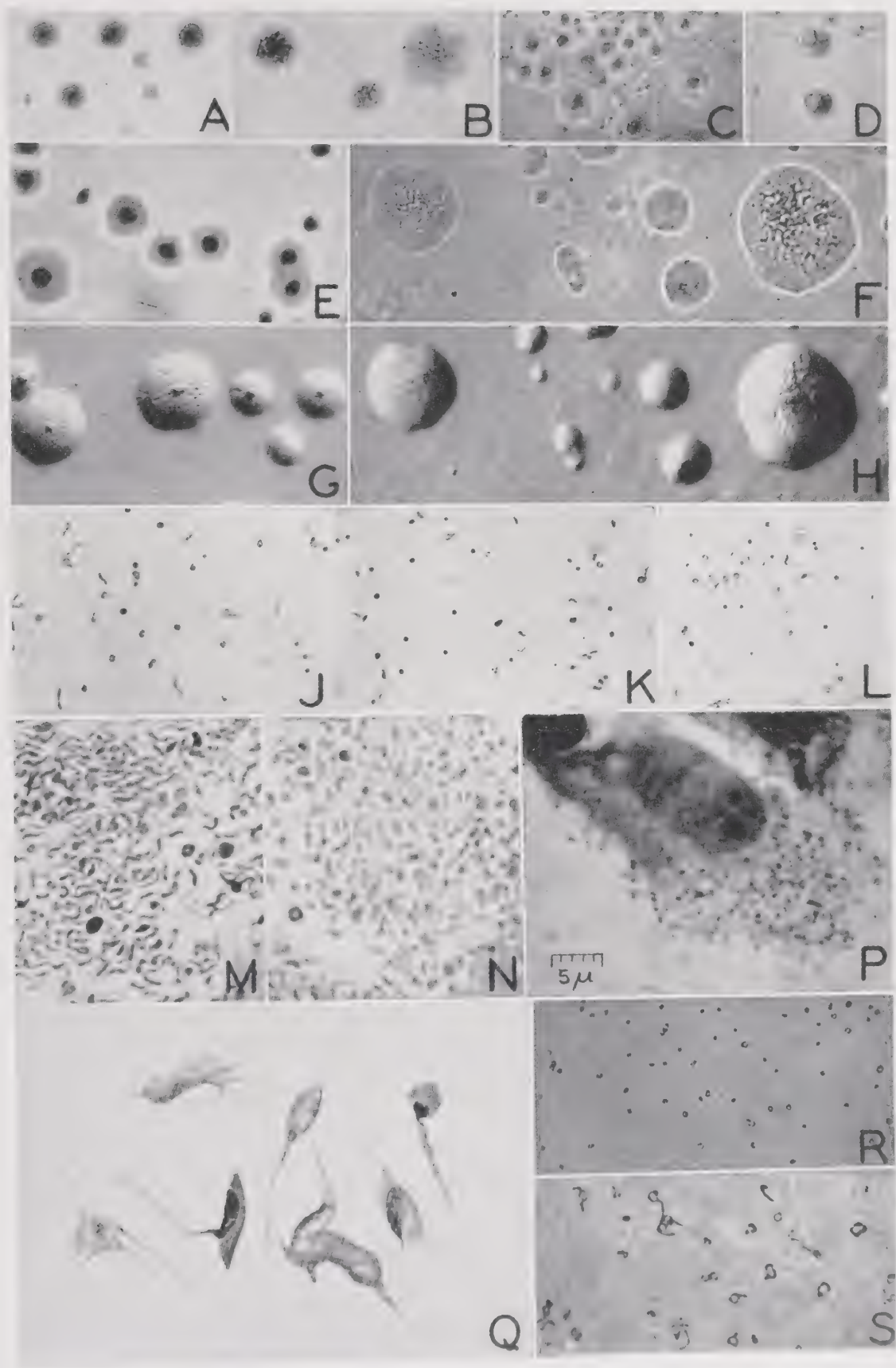


FIGURE 33



muscle infusion peptone broth or agar (2 per cent) at pH of 7.6 to 8.0, to which is added 30 per cent of filtered human ascitic fluid or of various animal sera (horse, bovine, rabbit). The addition of 0.5 per cent glucose can help in primary isolation and in obtaining a heavier growth in fluid media, but subcultures may then have to be made more promptly since the lowering of the pH to about 7.0 or less results in cessation of growth, and death of the culture. Growth in fluid media, which for most members of the group is optimum under aerobic conditions, may be associated with such slight turbidity, that its presence may be suspected only by comparison with an uninoculated tube of medium which should always be incubated along with the inoculated tubes. In primary isolations, when the added tissue or exudate by itself gives rise to appreciable turbidity, it is best to subculture about 0.2 cc. into fresh fluid medium as well as on agar plates on the fourth day, and again on the seventh day if necessary. Subculture at these intervals, even when the original medium is clear, may be practiced as a form of "blind passage" which might ultimately yield visible growth. In early cultures the first evidence of growth or slight turbidity may not appear for 3 to 14 days, but growth may become apparent in 24 to 48 hours after several serial passages. Transmissible turbidity in the absence of visible bacteria in Gram-stained smears can be proved to be due to pleuropneumonia organisms by: (1) the development of the characteristic, minute colonies when the fluid culture is seeded on solid medium containing the same kind of protein, and (2) by demonstrating the characteristic polymorphic structures in Giemsa-stained smears.

Inoculation on 30 per cent serum or ascitic fluid agar is the method of choice for primary isolation from infected tissues or exudates. Colonial development occurs best when evaporation of the medium is prevented by sealing the Petri dish. The

minute colonies may require as little as 2 days' or as much as 7 days' incubation before they can be detected with the aid of a hand lens or the low power of the microscope. Isolated colonies appear distinctly outlined and slightly elevated, with either a nipplelike, darker center or a vacuolar meshwork on the surface. They are 10 to 600  $\mu$  in size, depending on the species and conditions of growth (Fig. 33A to H). Subculture is best accomplished by cutting out a piece of agar containing such colonies and streaking it across another agar plate. When all colonies do not appear the same (except for size) it is advisable to cut out single colonies for subculture, for in this manner three distinct immunologic types were demonstrated in a culture from the nasal mucosa of a single mouse (Sabin and Johnson, 1940). Growth in fluid media can be obtained by dropping pieces of agar with many colonies into tubes of fluid medium containing the same kind of serum or ascitic fluid as the solid medium. Growth may become apparent in a few days in the form of granules or flakes close to the piece of agar, and only rarely as a diffuse turbidity; 6 to 10 rapid serial subcultures may be needed before the granular type of growth changes to one of diffuse turbidity. Organisms of the pleuropneumonia group can grow on the chorio-allantoic membrane of chick embryos, although at least one of the mouse strains (Type A) will grow well only on chorio-allantoic membranes of embryos that have been chilled to death at 4° C. prior to incubation. Growth of parasitic members has also been obtained in media without serum, consisting of Tyrode's solution in which were suspended small amounts of minced mouse- or chick-embryo tissues, but multiplication appeared to be predominantly intracellular (Sabin, 1941).

The filtration behavior of pleuropneumonia organisms differs from that of viruses, since the elementary bodies of the former constitute only a small proportion of the total number of reproductive units

present in a culture, while the larger units are retained by membranes of very large average pore diameter. Properly performed, gradocol membrane-filtration tests with various members of the pleuropneumonia group have yielded values in the range of 125 to 250  $m\mu$ , which is of the same order of magnitude as the size of many of the larger viruses. At least one of the members of this group, the Type-A organism of mice, has been shown to produce a true thermolabile, antigenic exotoxin in cultures (Sabin, 1941). A micro-organism recently recovered from experimentally inoculated chick embryos has been shown to elaborate a heat-stable, nonantigenic substance which causes edematous pulmonary consolidation and death by nasal instillation in cotton rats. Cultures of the same organism from chick embryos were also found to agglutinate erythrocytes of chickens and other species. Hemagglutination was specifically inhibited by homologous hyperimmune rabbit serum and by a high percentage of sera from hens from the hatchery which furnished the eggs from which the organism was isolated (van Herick and Eaton, 1945).

The pleuropneumonia organisms which occur in unrelated species of animals are immunologically distinct, and multiple immunologic types have also been found in the same species. Tests on a micro-organism of human origin failed to reveal a common antigen with any of the mouse and rat strains (Warren and Sabin, 1942), and Dienes (personal communication) reports that the many strains which he has now isolated from patients are not serologically homogeneous.

#### DISTRIBUTION IN NATURE AND RANGE OF PATHOGENICITY

In general, the parasitic members recovered from human beings, cattle, sheep and goats, dogs, rats, mice, and possibly also from chicken eggs, appear to be limited in distribution to hosts of the same or very

closely related animal species. Thus, the organism of bovine pleuropneumonia is limited to cattle, while that of agalactia has been found only in sheep and goats.

As far as is known now, the micro-organisms cultured from human material are indigenous to man. The normal human female genital tract appears to be a natural habitat, while, according to information available thus far, their presence in the male genito-urinary tract appears to be associated with pathologic disturbances. There is suggestive evidence of their presence in 2 to 5 per cent of human tonsils (Sabin and Johnson, 1940; Sabin, 1941), and certain observations of Seiffert (1937) and Dienes (personal communication) suggest that they occur in the sputum of patients with bronchiectasis or other chronic lung infections. Whether or not organisms of this group can exist in intimate symbiosis with certain bacteria is still a matter of controversy, particularly as regards *Streptobacillus moniliformis* (Klieneberger, 1942). However, Brown and Hayes (1942) have shown that cultures of several seemingly pure strains of gonococcus were associated with pleuropneumonia organisms; by taking advantage of the fact that sulfadiazine inhibits the growth of the gonococcus but not of the pleuropneumonia group, they were able to obtain pure cultures of the latter, which upon continued cultivation did not revert to the gonococcus.

#### PATHOGENESIS

Many, if not all, of the members of the pleuropneumonia group, although capable of multiplying in cell-free media in vitro, are intracellular parasites in vivo and exhibit special affinities for mesenchymal cells. Of special significance also is the demonstration that during the multiplication of one of the mouse micro-organisms (Type A) in the mesenchymal cells of the pleura and peritoneum, a specific neurotropic exotoxin is liberated which produces extensive degen-



erative lesions without inflammatory reaction in the cerebellum and elsewhere in the nervous system, with resulting choreiform nervous signs. The pathologic changes produced by the different members of the group vary all the way from no local lesions, as in the case of the Type-A mouse micro-organism growing in the peritoneum and pleura, to extensive pyogenic lesions as produced wherever the L<sub>4</sub> micro-organism of rats multiplies. However, except in the case of the L<sub>4</sub> micro-organism, suppuration is extremely rare, and an attack on the connective tissue elements associated with subacute inflammation and proliferation is the more common pathologic picture.

### DIAGNOSIS AND TREATMENT

To be included in the pleuropneumonia group a micro-organism must possess the characteristics described above, and this cannot be determined merely by an examination of colonies on solid media. Certainly organisms possessing ordinary bacterial morphology and distinctly stained by the Gram method, e.g., *Streptobacillus moniliformis*, or the organism recovered from a human case of subacute bacterial endocarditis and called pleuropneumonia-like (Herschberger et al., 1945; Wallerstein et al., 1946), etc., should not be included in the group. While the colonial morphology can constitute the first clue, colonies should be subcultured on solid media and then in liquid media to make certain that no bac-

terial forms grow out and that the structures present correspond to those of the pleuropneumonia group. Sulfonamides (up to 2 mg. per cc.) and penicillin (about 100 units per cc.) have no effect on the growth of the pleuropneumonia group and may be incorporated in the medium (Salaman, 1946). Attempts to determine by serologic methods and skin tests whether the organisms recovered from human material are etiologically related to the chronic urethritis or prostatitis, arthritis or Reiter's syndrome with which they were associated have yielded negative results (Dienes, personal communication). It should be noted, however, that the development of antibodies in various susceptible hosts infected with members of the pleuropneumonia group is either irregular or indefinite (Sabin, 1941).

Stovarsol (acetylamino-hydroxy-phenyl-arsonic acid) has been reported to exert both a preventive and curative effect on agalactia contagiosa. Gold compounds exert a curative effect on the chronic arthritis produced by the Type-B mouse micro-organism, even though they are without effect in cultures in vitro (Sabin and Warren, 1940). The effect of streptomycin must still be regarded as not established, since in the single available report on the subject (Powell et al., 1946) a micro-organism was used whose recorded properties do not justify its inclusion in the pleuropneumonia group. As already mentioned, sulfonamides and penicillin are without effect.

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## 31

# The Actinomycetes

### INTRODUCTION

The actinomycetes are a varied group of filamentous micro-organisms with characteristics intermediate between those of bacteria and molds, growing in the form of a branched mycelium. They range from the strictly parasitic, nonobligatorily anaerobic *Actinomyces* proper, which are bacteriallike in their principal characteristics, to the moldlike *Streptomycetaceae*. Members of the group of medical interest are the anaerobic *Actinomyces bovis*, the causative agent of actinomycosis in animals and in man, and several aerobic species of *Nocardia* which cause nocardiosis in animals and man.

### HISTORY

*Actinomyces bovis* was named and described by Harz in a study of material from "lumpy jaw" in cattle, a disease to which Harz also applied the name actinomycosis (Bollinger, 1877). Israel (1878) identified this disease with actinomycosis in man, and Wolff and Israel (1891) first cultivated the organism, adequately characterized it, and demonstrated its pathogenic action.

The first pathogenic aerobic actinomycete was described from "farcin du boeuf" in cattle by Nocard (1888) and was later named *Nocardia farcinica* by Trevisan (1889). The first pathogenic aerobic actino-

mycete found in a human infection, pseudo-tuberculosis with brain abscesses and meningitis, was described by Eppinger (1890) as *Cladothrix asteroides* and later named *Nocardia asteroides* by Blanchard (1896).

### THE PARASITIC ACTINOMYCETES (GENUS ACTINOMYCES)

#### HABITAT AND SOURCES

The true *Actinomyces* are found in the lesions or exudates of actinomycosis of man, cattle and other animals, and also, in the absence of such disease, between the teeth and gums of man, in the tartar on teeth, in the crypts of the tonsils, and perhaps on other mucous membranes. They have not been found in nature apart from a parasitic habitat.

#### MORPHOLOGY AND VARIATION

The *Actinomyces* are Gram-positive, branching, filamentous organisms. They are not acid fast, do not form spores, and are nonmotile. The individual filaments are less than 1 micron wide and have a marked tendency to fragment into bacillary or single-branched "twiglike" elements. In the

tissues under pathologic conditions, in fluid media, and in the depths of solid media, the more characteristic rough varieties tend to grow as compact whitish mulberry-shaped colonies, or as tiny crumblike masses, each of which consists of a branched mycelium. In the "sulphur granules" that may occur in lesions or exudates, the peripheral ends of filaments may be swollen to form the eosinophilic "clubs" whose radial arrangement is responsible for the name actinomycete ("ray-fungus"). In agar surface colonies, rough strains produce dead white or pale yellowish, irregular, "heaped-up" colonies, not exceeding about 2 mm. in diameter. Microscopic preparations from such colonies show branching filaments only when care is taken to prevent fragmentation; otherwise, short, bent, and single-branched forms are the rule, often with swollen ends and in V- and Y-groupings like the diphtheria bacillus. Smooth strains, which may develop in cultures from rough ones, or may be recovered directly from the human mouth and are common in actinomycosis of animals, show white lustrous colonies that may be contoured or dome-shaped, and contain cells whose morphology ranges from a rough diphtheroid form down to regular unbranched Gram-positive rods.

#### CULTIVATION AND BIOCHEMICAL REACTIONS

Growth appears at 37° C. in from 3 to 6 days in infusion broth or agar, pH 7.2-7.6, in the presence of 1 per cent of dextrose, preferably under anaerobic conditions. Brain-heart infusion medium containing 2 per cent of agar is useful for plating. Chopped-meat infusion broth or thioglycolate-agar broth may also be used. Strains can best be maintained if transferred alternately to different media. The organism has a limited tolerance for oxygen which varies with the strain. While some strains grow aerobically on the surface of agar media, their preference for reduced oxygen tension

is shown by their tendency to grow only in the depths of dextrose agar shake cultures, frequently as a concentrated zone of colonies 0.5 to 2 cm. below the surface. Maintenance of cultures is best assured by growth under anaerobic conditions.

Growth occurs in the absence of carbohydrate but is more abundant in its presence.

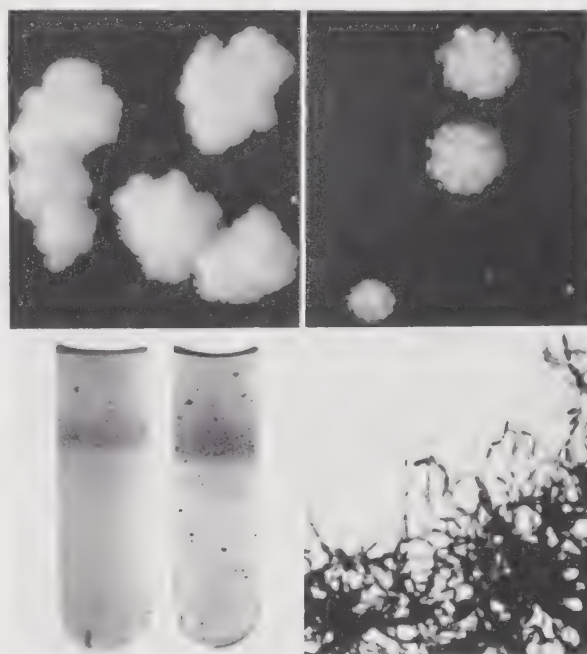


FIG. 34. (Top, left and right) Typical rough surface colonies of *A. bovis* on brain heart agar streaked plates incubated anaerobically with 5% CO<sub>2</sub> for 6 days at 37° C.  $\times 8.7$ . (Bottom, left) Shake cultures of *A. bovis* in dextrose agar, incubated aerobically at 37° C. for 4 to 6 days. (Bottom, right) Gram-stained films of *A. bovis*.  $\times 865$ .

Acid without gas is produced from dextrose and other hexoses. The fermentation of disaccharides, polysaccharides and alcohols varies with the strain. Most strains are non-proteolytic, do not form indol or reduce nitrate, and are nonhemolytic; some strains may form H<sub>2</sub>S. Neither pigments nor antimicrobial substances have been found in true *Actinomyces*. The organisms are killed within one hour at 60° to 65° C. They may remain alive for several months when dried in vacuum and stored in the refrigerator



## ATTEMPTS AT CLASSIFICATION

Strains of *Actinomyces* isolated from animals and man, from lesions or normal mucous membranes, show wide variations in morphology, growth rate, oxygen tolerance and fermentation reactions, and are somewhat heterogeneous serologically; but these differences have not been consistent enough for purposes of classification. The parasitic actinomycetes may therefore be grouped provisionally in a single species, *Actinomyces bovis* (synonyms: *A. israeli*; the "Wolff-Israel type" of actinomycete).

## PATHOGENICITY

Single inoculations of cultures into guinea pigs, rabbits or other animals by any of the common routes frequently produces no effect, but for undefined reasons results irregularly in the development of nodules, from which the organisms can sometimes be recovered, and in which sulphur granules can only occasionally be found. This inconsistent pathogenicity has not generally been increased by animal passage, by traumatization during inoculation (e.g., with a splinter or other foreign body), or by addition of other bacteria to the inoculum. Progressive actinomycosis, with sulphur granules and typical pathologic signs, has been produced—although again without regularity—by repeated inoculation at intervals that might have permitted development of allergy to the organism. While these findings as a whole leave no doubt that *A. bovis* is of itself capable of causing typical actinomycosis, they also point to the existence of as yet undefined factors in the pathogenesis of the disease.

## ACTINOMYCOSIS

## DEFINITION AND CLINICAL FEATURES

Actinomycosis is a subacute or chronic, generally progressive disease of man, cattle, swine, horses and other animals, character-

ized by the development of indurated granulating swellings chiefly in connective tissue, by suppuration usually of limited extent, and by the presence in the pus or lesions of *Actinomyces bovis*, demonstrable microscopically or culturally. In man the lesions are found chiefly in the cervicofacial connective tissues and in the thoracic or abdominal viscera, and develop over periods ranging from a few weeks to a year or more. The lesions spread widely by contiguity, sometimes pointing toward the skin and forming fistulae that tend to heal and reform elsewhere; rarely pointing toward mucous or serous membranes. The organism may be disseminated through the blood, or, in the lungs, through the bronchi. The lymphatic system is only rarely involved. Bone may be eroded in the path of the lesion, but is seldom affected interstitially except in the jaws.

**Cervicofacial** actinomycosis accounts for more than half of all cases in man. It apparently originates from the mouth, but affects the soft tissues and skin of face and neck, and the tongue, and, secondarily, the maxillary bones. The salivary glands, larynx, thyroid and lachrymal glands, the orbit and even the brain may more rarely be involved. The commonest lesions appear on the cheek or submaxillary skin, and are characterized by indurated or edematous swellings, bluish or reddish in color, with a tendency to form a series of irregular folds separated by furrows, the healing lesions forming scars as new lesions develop.

**Thoracic** actinomycosis accounts for about 15 per cent of human cases. It is found mainly in the lungs, with the formation of abscesses and cavities which are usually small. Extensive lesions may be found in the bronchi, and their rupture may lead to dissemination of the infection by way of the bronchial tree. Actinomycotic pleurisy and empyema have been observed, as has involvement of the heart and pericardium. Thoracic lesions may originate from the mouth or throat by aspiration, by

extension from the abdomen, or by metastasis.

**Abdominal actinomycosis** comprises about 20 per cent of human cases. The lesions may be found in any organ but are most common in the region of cecum and the appendix. From here they may extend with suppurating foci and the formation of fibrous adhesions to the abdominal wall, where skin lesions may appear similar to those of cervicofacial actinomycosis. Or the lesion may remain circumscribed, forming a fibromalike mass. The liver is commonly attacked, and lesions of the genital tract are relatively frequent. The stomach, small intestine and kidney are seldom affected. Infection is probably derived in most instances directly or indirectly from the intestinal or genital mucous membranes which, however, are not themselves involved.

**In the skin**, actinomycosis secondary to lesions of underlying tissues or organs is relatively common, as has been noted; but it is doubtful whether true actinomycosis is ever primary in the skin.

The most complete account of the clinical features of actinomycosis in English is that of Cope (1938).

#### HISTOPATHOLOGY

The microscopic appearance of the lesions of actinomycosis varies from that of an acute abscess with an abundance of polymorphonuclear cells to the more chronic lesion in which proliferating connective tissue is the most conspicuous feature. Commonly the picture includes necrosis with an abundance of leukocytes, surrounded in turn by granulation tissue and a profuse formation of dense fibrous tissue. It is thus not characteristic, unless sulfur granules are present. These are frequently lacking in either tissues or pus and when present, particularly in human lesions, may not show typical clubs. Moreover, club-bearing granules not easily distinguished from those of true actinomycosis are found in several dis-

tinct diseases (Rosebury, 1944). These are so rare in man, however, that diagnosis by demonstration of granules is hardly exceptional.

Details of the typical granule are best seen under magnifications of 400 diameters or more. It may be roughly circular or irregular in outline, or may consist of several colonies of different size and shape that have coalesced. It is composed of a dense reticulum of fibrils which take the violet dye in sections stained by Gram's method, but may otherwise stain irregularly. Around the periphery the ends of individual filaments may project, with or without radially arranged hyaline clubs. These take the eosin stain and are several times wider than the filaments whose ends they enclose.

#### EPIDEMIOLOGY

The theory of Bostroem (1891) that actinomycosis results from traumatization with grass, straw or grain carrying the infective agent has not been established. Davis (1941) has shown that the disease is not more common in rural than in urban groups, and that its history in the individual only rarely includes the likelihood of trauma from vegetable fibers. There is no clear evidence that actinomycosis is ever communicable. The concept of Wolff and Israel (1891), amplified by Wright (1905), has been well substantiated and has come to be generally accepted. According to this view, *A. bovis* is a true parasite of mucous membranes, never found in nature apart from a parasitic or pathogenic habitat; it occurs on mucous membranes, possibly in the absence of any disturbance, certainly in the presence of only low-grade non-specific inflammatory processes, e.g., of the gums. It produces disease as an endogenous infection comparable with subacute bacterial endocarditis due to *Streptococcus viridans*, or to peritonitis or cystitis due to *Escherichia coli*.



## PATHOGENESIS

Strains of *A. bovis* isolated from mucous membranes have been found capable, like those obtained from typical disease, of producing actinomycosis experimentally. Yet, as we have seen, experimental studies have thrown little light on the mechanism of the process. The comparative rarity of actinomycosis suggests that autoinoculation resulting from minor trauma, perhaps even when repeated, can be no more than a contributory incident. More severe traumatic events have often been associated with actinomycosis: e.g., tooth extraction or other injury to mouth or throat; human bite or knuckle injuries from a blow to the teeth; or aspiration of an extracted carious tooth or tooth fragment into the lungs. *A. bovis* occurs in salivary calculus or tartar, in the deposition of which it appears to play a part; and detached masses of tartar may be involved in comparable traumatic accidents. There is suggestive but inadequately confirmed evidence that sensitization from repeated inoculation may be a factor in the pathogenesis of the disease (Slack, 1942; Rosebury et al., 1944).

## DIAGNOSIS

A clinical diagnosis of actinomycosis can at present be established only by demonstration of *A. bovis* microscopically, or preferably, by cultivation. Attempts to apply serologic reactions or allergic skin tests have been unsuccessful. Direct microscopic demonstration depends on the presence of Gram-positive branched rods or filaments, with or without clubs, or on the observation of typical sulfur granules in tissue sections. Isolation of the causative agent is the most convincing diagnostic procedure. Where exudate from closed lesions appears to be free from contamination, inoculation, using crushed granules if present, into 1 per cent dextrose infusion agar shake tube cultures may suffice. Several tubes should be

inoculated serially and incubated aerobically at 37° C. for 3 to 6 days. Successful cultures show whitish spherical or mulberry-shaped colonies, up to 3 mm. in diameter, growing only in the depths of the agar, often with a dense zone of colonies 0.5 to 2 cm. below the free surface. Diagnosis is confirmed by demonstration in such a colony, removed with a capillary pipette, of a branched mycelium or of twiglike Gram-positive rods and short filaments.

Where the exudate is likely to be contaminated, as in sputum or material from draining lesions, Rosebury et al. (1944) have recommended serially streaked plates on Bacto brain-heart medium containing 2 per cent of agar. The plates are incubated anaerobically for 4 to 6 days at 37° C. The opaque, dead white or pale yellowish, heaped-up colonies of *A. bovis* can be identified without great difficulty even in the presence of abundant contaminating growth; but since other bacteria may produce similar colonies, it is essential that they be examined microscopically. A typical colony should be fished to dextrose-agar shake cultures for confirmation as described above. It may be noted that recovery of an occasional *A. bovis* colony from materials subjected to direct contamination from a mucous membrane (e.g., in expectorated sputum) must be interpreted with caution because of the frequent presence of the organism on the mucous membranes.

## CHEMOTHERAPY

Before the advent of modern chemotherapy many forms of treatment, including iodides, thymol, copper or iron salts, arsphenamines, vaccine therapy, X-rays, radium and surgical drainage, for actinomycosis were attempted with varying results. Results obtained with sulfonamides were promising but somewhat inconsistent (Ladd and Bill, 1943). Good results have been obtained with penicillin, particularly when large doses (80,000 to 120,000 units) were

given intramuscularly (McCrea et al., 1945). Different strains of *A. bovis* have been found to differ in sensitivity to penicillin in vitro. It has been suggested that sulfadiazine may be more useful than penicillin in some cases (Dobson and Cutting, 1945), and that surgical drainage or excision is an indispensable adjunct to penicillin therapy in thoracic actinomycosis (Poppe, 1946).

### SAPROPHYTIC \* (AEROBIC) ACTINOMYCETES (GENUS NOCARDIA)

#### HABITAT AND SOURCES

Species of *Nocardia* are found in lesions of man and animals, and, unlike *Actinomyces*, are free living in nature, having been described as air-borne laboratory contaminants (Henrici and Gardner, 1921) as contaminants on the skin of healthy guinea pigs (Gaiginsky, 1934) and as soil inhabitants and plant disease "potato scab" organisms (Gordon and Hagan, 1936).

#### MORPHOLOGY AND VARIATION

The *Nocardia* are Gram-positive, partially acid-fast or nonacid-fast, branching, filamentous organisms, 1  $\mu$  or less in diameter. Some species fragment readily into bacillary and coccoid elements while other species have a tendency to remain filamentous. In tissues and in exudates from draining sinuses they may appear as yellowish-white, red or black, dense, tangled mycelial "granules" with or without clubs at the periphery. In spinal fluid, empyemic fluid, sputum and, occasionally, in pus, only bacillary forms may be seen. Not infrequently the acid-fast varieties may be mistaken for tubercle bacilli in stained sputum smears.

#### CULTIVATION

Species of *Nocardia* are aerobic organisms that grow readily at 37° C. or room tem-

perature on a variety of simple media such as beef infusion glucose agar, Sabouraud's glucose agar, Czapek's agar, etc. Colonies are slow growing, however, and it is necessary to wait for 3 to 4 weeks before a typical appearance is obtained in the so-called

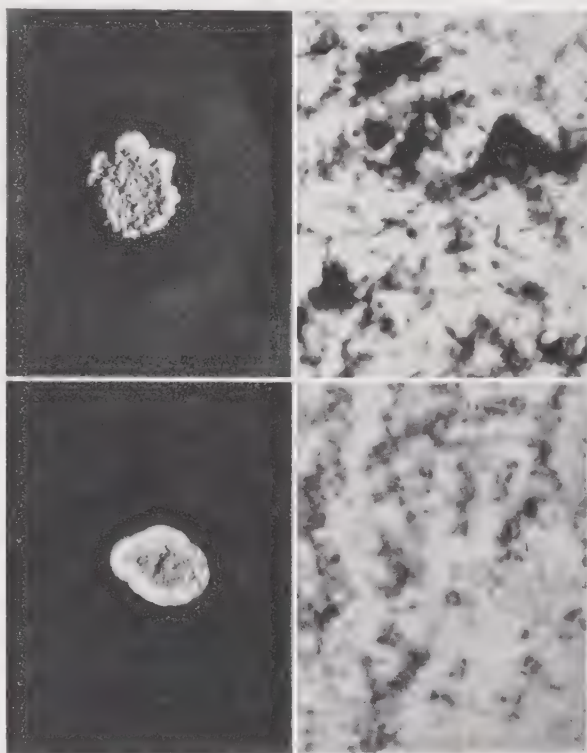


FIG. 35. (Top, left) *N. asteroides*. Colony on Sabouraud's glucose agar, 12 days old. (Top, right) *N. asteroides* from Sabouraud's agar. Gram stain.  $\times 970$ . (Bottom, left) *N. brasiliensis*. Colony on Sabouraud's glucose agar, 12 days old. (Bottom, right) *N. brasiliensis* from Sabouraud's agar. Gram stain.  $\times 970$ .

"giant cultures." Such cultures show a marked variation in odor, gross appearance, pigment production and texture on different media, and even on the same medium. On solid media the colonies are usually glabrous, wrinkled or granular and resemble closely the growth of some acid-fast bacilli. Occasional strains produce an aerial mycelium which gives the colony a chalky or powdery appearance, but, on transfer,

\* The term "saprophytic" is used to indicate that these actinomycetes can exist as free living forms on dead organic materials, although they also possess pathogenic properties.



## THE ACTINOMYCETES

TABLE 48. COMPARISON OF CULTURAL, MORPHOLOGIC AND STAINING REACTIONS OF SPECIES OF NOCARDIA

SPECIES	COLOR OF GRANULE	ACID FAST	SABOURAUD'S GLUCOSE AGAR	CZAPEK'S AGAR (PIGMENT)	FRAGMENTATION OF MYCELIUM
1. <i>Nocardia asteroides</i> (Eppinger) Blanchard, 1896 Syn. <i>Cladothrix asteroides</i> Eppinger, 1890 <i>Streptothrix carnea</i> Rossidoria, 1891 <i>Streptothrix Freei</i> Musgrave & Clegg, 1907 <i>Actinomyces gypsoides</i> Henrici & Gardner, 1921 <i>Actinomyces ast.</i> var. <i>serratus</i> Satory, Meyer & Meyer, 1930 <i>Proactinomyces ast.</i> var. <i>crateriforme</i> Baldacci, 1938 <i>Proactinomyces ast.</i> var. <i>decolor</i> Baldacci, 1938	Yellowish-white, with or without clubs	+	Glabrous, rarely chalky. Moist, soft, folded or wrinkled and granular. Yellow, orange-ochraceous, red	Yellow to orange-ochraceous	+
2. <i>Nocardia brasiliensis</i> (Lindenberg) Cast. and Chalmers, 1913 Syn. <i>Discomyces brasiliensis</i> Lindenberg, 1909 <i>Actinomyces mexicanus</i> Boyd & Crutchfield, 1921	Yellowish-white, with or without clubs	+	Frequently chalky. Folded, cerebriform, tenacious and dry. Earthy odor. Yellow, orange-ochraceous	Yellow to orange-ochraceous	+
3. <i>Nocardia madurae</i> (Vincent) Blanchard, 1896 Syn. <i>Streptothrix madurae</i> Vincent, 1894 <i>Nocardia indica</i> Chalmers & Christopherson, 1916 <i>Actinomyces micetomae</i> Greco, 1916 <i>Discomyces bahiensis</i> Piraja da Silva, 1919	Yellowish-white, with or without clubs	—	Glabrous. Moist, soft, wrinkled. Cream colored	Cream colored at first; later becoming pinkish to red	—
4. <i>Nocardia Pelletieri</i> (Laveran) Pinoy, 1912 Syn. <i>Micrococcus Pelletieri</i> Laveran, 1906 <i>Nocardia africanus</i> Pijper & Pullinger, 1927 <i>Nocardia Genesisii</i> Fróes, 1930	Red, with or without clubs	—	Small. Glabrous, heaped, wrinkled. Mucilaginous. Coral pink to red	Coral red	—
5. <i>Nocardia paraguayensis</i> (Almeida) Conant, 1947 Syn. <i>Actinomyces paraguayensis</i> Almeida, 1940	Black, with clubs	—	Glabrous. Soft, white center. Projecting border adherent, darker	Dark cream	—

this character may be lost. Pigmentation is produced best and is more constant on Czapek's agar and varies, depending upon the species, from cream to yellow, orange ochraceous, and pink to coral or brick red. The texture may be soft, moist and muc-



FIG. 36. (Top, left) *N. madurae*. Colony on Sabouraud's glucose agar, 12 days old. (Top, right) *N. madurae* from agar. Gram stain.  $\times 970$ . (Bottom, left) *N. Pelletieri*. Colony on Sabouraud's glucose agar, 12 days old. (Bottom, right) *N. Pelletieri* from Sabouraud's agar. Gram stain.  $\times 970$ .

laginous, or hard, dry and granular. On liquid media the species develop wrinkled surface pellicles with the medium remaining clear. A comparison of the cultural appearance, pigment production, staining reaction and morphology of 5 species of *Nocardia* pathogenic for man is presented in Table 48.

#### BIOCHEMICAL REACTIONS

A few of the biologic properties of the aerobic actinomycetes remain constant

when different species or several strains of the same species are tested; they do not ferment carbohydrates and do not produce indol,  $H_2S$  or  $NH_3$ . There is variation of reaction among strains studied by a single investigator and between single species studied by different investigators concerning coagulation of milk, reaction in litmus milk, reduction of nitrates and the ability to liquefy gelatin. Among 18 strains of *Nocardia brasiliensis*, Lacaz (1945) found 9 to liquefy gelatin, 9 to coagulate milk, 3 to produce an acid reaction, 4 an alkaline reaction, and 11 no reaction in litmus milk; 3 strains reduced nitrates to nitrites. Ten strains of *Nocardia mexicanus* and 3 strains of *N. brasiliensis* reported by González Ochoa (1945) to be identical were found to liquefy gelatin and coagulate milk. A comparison of the biologic activities of 5 species of *Nocardia* is presented in Table 49.

#### ATTEMPTS AT CLASSIFICATION

The genus name *Nocardia* should be used for these organisms according to the classification of Waksman and Henrici (1943). Because of the wide variation of behavior, both culturally and biologically, among strains of pathogenic aerobic actinomycetes, several different species have been described. Comparative studies of several strains by González Ochoa (1945, 1947) and Lacaz (1945) have shown that many of these should be reduced to synonymy with previously described species. The methods of study proposed by Waksman (1919, 1920) results in the differentiation of the 5 species listed in Table 48.

#### PATHOGENICITY

*Nocardia asteroides* is the only species showing pathogenic properties for laboratory animals. This species, however, varies greatly in its ability to produce infection (Drake and Henrici, 1943). Only rarely can a progressive disease be produced in ani-



TABLE 49. BIOLOGIC ACTIVITIES OF SPECIES OF NOCARDIA

SPECIES	CARBO- HYDRATES	INDOL	H <sub>2</sub> S	NH <sub>3</sub>	GELATIN LIQUE- FACTION	MILK COAGU- LATION	LITMUS MILK	NITRATES TO NITRITES
1. <i>N. asteroides</i> .....	—	—	—	—	—	—	Alk.	+
2. <i>N. brasiliensis</i> .....	—	—	—	—	+	+	—	±
3. <i>N. madurae</i> .....	—	—	—	—	+	+	Acid	+
4. <i>N. Pelletieri</i> .....	—	—	—	—	+	+	Acid	+
5. <i>N. paraguayensis</i> ..	—	—	—	—	+	+	Acid	+

mals. Rabbits injected intravenously with a sufficiently large inoculum develop a generalized infection with the production of miliary abscesses throughout the entire body. Intramuscular and subcutaneous injections result in local abscesses only. These resorb or rupture spontaneously and heal with no extension of the infection. Guinea pigs injected intraperitoneally usually show a diffuse peritonitis with abscess formation on the peritoneal surface. Death is caused more from a toxic effect of the inoculated material than from extensive invasion by the fungus.

#### ALLERGIC PROPERTIES

It is not known whether an infection with aerobic actinomycetes in humans causes hypersensitivity demonstrable by an injection of the organisms or their products. Drake and Henrici (1943) demonstrated that rabbits and guinea pigs infected with *N. asteroides* do give a specific delayed reaction to a protein and polysaccharide fraction of *N. asteroides* and to extracts of the powdered organisms and that these materials do not cause reaction in tuberculous animals. No reaction was obtained with "asteroidin," prepared from broth cultures in the same manner as Old Tuberculin, with culture filtrates or with extracts of heated organisms. Since Drake and Henrici could not extract a protein from the medium in which the organism was grown, it is not surprising that culture filtrates gave no reactions. In

contrast, filtrates from cultures of other fungi (coccidioidin, blastomycin, histoplasmin) do elicit reactions in guinea pigs infected with the corresponding organism. Drake and Henrici (1943), however, have shown that the whole cells and lipid fractions of *N. asteroides* produce reactions in normal animals.

### NOCARDIOSIS

#### DEFINITION AND CLINICAL FEATURES

Nocardiosis is a chronic suppurative, purulogranulomatous disease of the subcutaneous tissues and bones (Mycetoma) characterized by multiple tumefactions and draining sinuses from which "granules" (yellowish-white, red or black) are expressed in the pus or found in the tissues; or, a pseudotuberculous infection (systemic) of the lungs and pleura with hematogenous spread throughout the body, especially to the brain and meninges, in which filamentous, bacillary or coccoid, acid-fast forms may be found in the sputum, spinal fluid, or pus from subcutaneous abscesses.

Mycetoma of the extremities results in the clinical picture of Madura Foot, although other subcutaneous tissues of the body also may become infected. The characteristic lesion with pain, swelling, and sinus formation, and eventual clubbing and marked deformity of the infected member is developed only after months or years. Infection spreads by extension through adjacent tissues with bone destruction, mul-

multiple abscesses which rupture, and with no systemic reaction unless secondary bacterial invasion is established. Histologically, sections of the sinus and abscess walls may

depend on the presence of granules, surrounded by polymorphonuclear neutrophils, centrally located in the abscesses.

Systemic nocardiosis is caused by *N. asteroides* and is chiefly pulmonary in origin. Of 34 cases, including 2 new cases, reviewed by Kirby and McNaught (1946) the lungs were infected in 29 and, of these, 11 had metastases to the brain. Occasionally the presenting symptoms of headache, nausea and vomiting may indicate either brain tumor or brain abscess; or, the symptoms may be those of an infectious meningitis (tuberculous) with minimal or no findings in the lungs. Symptoms referable to a pulmonary infection include general malaise, fever, productive cough with sputum, night sweats, anorexia and loss of weight. Roentgenograms of the lungs usually show a progressive infiltrative process which may lead to multiple cavity formation. Hematogenous spread results in metastatic lesions throughout the body. Histologically, such lesions may be of a purulent nature, containing centers composed of polymorphonuclear neutrophils and a few mycelial fragments, or such areas may show a more advanced granulomatous reaction leading to granulation tissue, giant cells and scarring.

#### DIAGNOSIS

Lesions of the subcutaneous tissues producing the clinical picture of mycetoma should suggest infection by actinomycetes or infection by some of the higher fungi or molds (Maduromycosis). Pus from the draining sinuses, scrapings from the sinus walls and biopsy sections should be examined for actinomycotic granules. Fresh preparations of the pus and scrapings should be prepared by placing a drop of the material on a microscopic slide and covering the preparation with a cover glass. Microscopically, the granules appear as amorphous, lobulated masses surrounded by pus cells. The mass is composed of delicate ( $1\ \mu$  in diameter) tangled hyphae which

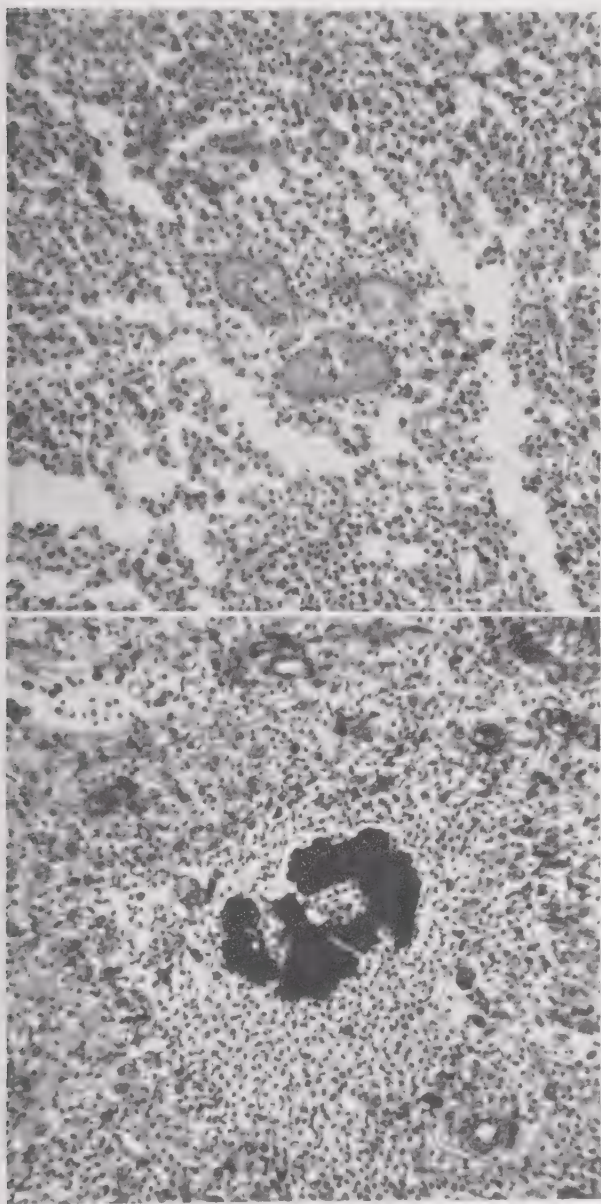


FIG. 37. (Top) Granules of *N. asteroides* in subcutaneous tissue.  $\times 147$ . (Bottom) Granule of *N. pelletieri* in subcutaneous tissue.  $\times 147$ .

show only a chronic inflammatory reaction. Further development of the acute purulent abscess results in a surrounding layer of granulation tissue infiltrated with round cells and fat-laden macrophages enclosed by a fibrous capsule. Diagnosis, however, de-



may or may not be terminated by sheaths (clubs) at the periphery. Such granules should be crushed and Gram stained. When examined, the smear contains short branching forms, bacillary and coccoid elements which are Gram positive. Cultures are obtained by inoculating blood agar plates to be incubated at 37° C., and Sabouraud's

sputum and pus. Rabbits may be injected intravenously and guinea pigs intraperitoneally with material suspected of containing aerobic actinomycetes. Since the inoculum may not be heavy and since such laboratory animals tend to recover from an initial infection, some of the animals should be sacrificed within two weeks if lesions are to be found.

#### TREATMENT

Except in cases of Mycetoma of the foot, in which amputation has proved successful, infections caused by aerobic actinomycetes have had a high mortality rate. Of the 34 cases reviewed by Kirby and McNaught (1946) 30 were dead at the time of publication. These authors pointed out, however, that as diagnosis had been made only at necropsy in 21 cases and that the organism demonstrated late in the disease in 13 cases, there was no opportunity to evaluate any form of treatment. Benbow et al. (1944) reported successful treatment of two patients infected with *N. asteroides* by surgical drainage, sulfonamides, iodides, roentgen therapy and supportive therapy; Kirby and McNaught (1946) found sulfadiazine to be effective, though applied too late, in one of their cases. It appears, therefore, that the sulfonamides offer an effective treatment if early diagnosis is made.

#### EPIDEMIOLOGY

Pathogenic aerobic acid-fast and non-acid-fast actinomycetes are free living in nature (Gordon and Hagen, 1936) and cause disease by air-borne contamination or by introduction into tissues through trauma. This view is substantiated by the study of systemic cases in which pulmonary infection is the rule, and of those cases demonstrating subcutaneous infection with no systemic reaction. There have been no reports of infection from man to man or from animal to man.

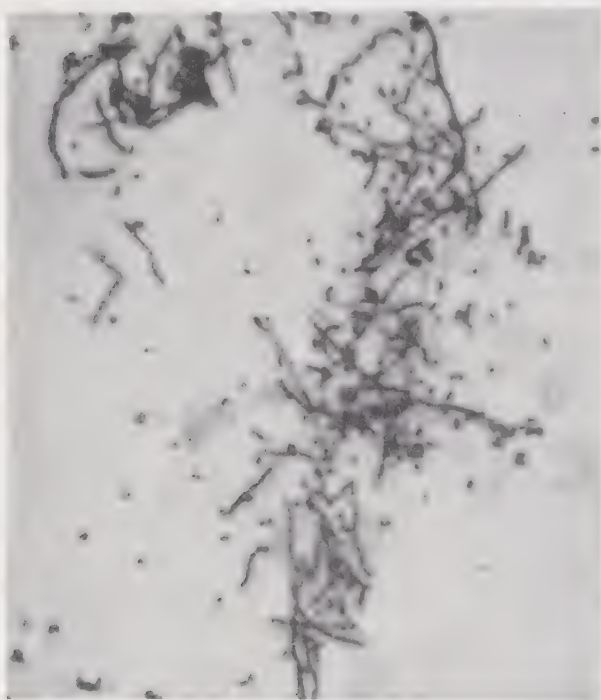


FIG. 38. Branching, bacillary and coccoid forms of *N. asteroides* in pus.  $\times 1740$ .

glucose agar slants to be incubated at room temperature, with the materials obtained from the lesions. Anaerobic cultures also should be prepared since *Actinomyces bovis* produces a similar granule. Sputum, and pus from subcutaneous abscesses, should be smeared and stained to demonstrate Gram-positive or acid-fast branching hyphae. Sputum, without concentration, and pus may be inoculated on blood agar plates and Sabouraud's glucose agar slants for culturing at 37° C. and room temperature, respectively. Spinal fluid should be centrifuged and the sediment stained for Gram-positive or acid-fast hyphae and cultured as for

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# 32

## Medical Mycology

### GENERAL

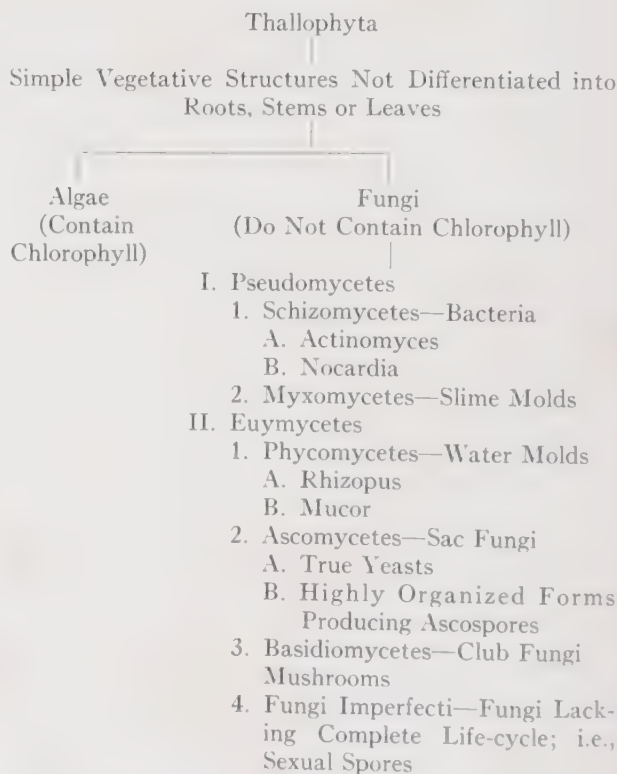
#### INTRODUCTION

As previously stated (page 576), the actinomycetes show a relationship not only to the single-celled bacteria but also to the higher filamentous fungi or molds. They exhibit branching like the higher forms, but their size is nearer that of the Eubacteriales. They are all slender organisms, their vegetative structures being one micron or less in diameter. Also, their methods of reproduction are essentially those of the bacteria since they fragment readily into bacillary or coccoid elements; or, as found among the Streptomycetaceae, they produce spores in the filaments as do the sporulating bacilli. Because of these characteristics, the actinomycetes have been placed in the Schizomycetes in a position intermediate between the Eubacteriales, or true bacteria, and the Eumycetes, or true fungi.

The true fungi, on the other hand, are characterized by the formation of filaments or *hyphae* which branch and intertwine to form a dense mat of growth, the *mycelium*, which represents the colony of the fungus. Produced on or from the mycelium are various types of reproductive bodies, or *spores*. This matlike growth made up of the mycelium with its spores constitutes an irregular rudimentary plant not differentiated into roots, stems or leaves, is called the *thallus*. Plants characterized by this structure are classified in the *Phylum Thallophyta*. The fungi constitute one of the two groups of plants in this Phylum. The other group, the algae, are distinguished from the fungi by the presence of chlorophyll in the plant body, whereas the fungi lack this material and are either saprophytic or parasitic.

The relationship of the various groups to be found in the Thallophytes is shown in Chart 14.

CHART 14



Fungi known to be pathogenic for man and animals are found in only two of the groups shown in Chart 14, namely, Schizomycetes, which contain the genera *Actinomyces* and *Nocardia*, and the *Fungi imperfecti*, which contain practically all other known pathogens.

#### CLASSIFICATION

The fungi, with few exceptions, do not lend themselves to the use of bacteriologic

technics for their identification. They are all Gram positive. They do not ferment sugars, do not reduce nitrates, cannot be characterized by tests such as coagulation of serum or milk or the liquefaction of gelatin, etc. They are identified, therefore, by their morphology: macroscopically, by the type of colony formation; and, microscopically, by the type of spores produced on or from the mycelium. Macroscopically, there are three colony types: yeast, yeastlike and filamentous. The yeast colony is soft and bacterial-like and is composed of single-celled, budding forms (*Cryptococcus* and *Saccharomyces*). The yeastlike colony is also soft and bacterial-like but is composed not only of single-celled, budding forms on the surface of the medium but also of hyphae which penetrate the medium (*Candida* species). The filamentous colony presents the appearance of a typical mold and is composed of branching hyphae, some of which penetrate the medium, *vegetative* mycelium, and some of which project from the surface, *aerial* mycelium. The aerial mycelium is referred to as a *reproductive* mycelium when spores are produced by the hyphae. The type of spore produced and the method of its formation, together with the type of colony, provide the morphologic characteristics by which fungi are identified.

The several types of spores produced by fungi may be divided into the two categories shown below.

#### SPORE TYPES

##### I. Sexual Spores—produced as a result of nuclear fusion.

1. *Ascospores*—spores produced in a sac, the ascus; found in the Ascomycetes.
2. *Basidiospores*—spores produced from a club-shaped structure, the basidium; found in the Basidiomycetes.
3. *Zygospores*—spores produced by fusion of two identical cells; found in the Phycomycetes.

4. *Oospores*—spores produced by fusion of two unlike cells; found in the Phycomycetes.

##### II. Asexual Spores—produced in, on, or by the mycelium without nuclear fusion.

1. *Thallospores*—spores produced by changes in the mycelium or thallus.
  - A. *Blastospores*—spores produced by budding; *Saccharomyces*, *Candida* and *Cryptococcus*.
  - B. *Chlamydospores*—spores produced by cells in the mycelium changing into thick-walled resistant structures; may be found in *all* fungi.
  - C. *Arthrospores*—spores produced by fragmentation of mycelium; *Geotrichum* and *Coccidioides*.
2. *True Conidia*—spores supported by a definite structure, the conidiophore; found among the Imperfecti.
3. *Sporangiospores*—spores produced inside swollen structures on the ends of hyphae; Phycomycetes.

#### EXAMINATION OF FUNGI

Since the fungi can be identified only by the type and arrangement of their spores, preparations must be made that will allow examination of these structures. The usual smear and staining method used for the bacteria are not applicable to filamentous cultures, as they break up the hyphae, disperse the spores and prevent proper examination. A preliminary examination of a tube culture is possible by placing the tube on the microscope stage and using the low power objective on the top of the slant or along its edge.

Slide cultures for continuous microscopic examination are made by various methods. For example, sterile slides and cover glasses, under which inoculated warm agar is run, support growth when placed in a damp chamber (sterile Petri dish with moist toweling or filter paper). Development of the fungus from the edge of the agar may be followed microscopically. In such preparations growth is undisturbed and the arrangement of the spores can be carefully studied.

Routine examination of yeast or yeast-



like cultures should be made by emulsifying some of the growth in a drop of water under a cover glass. Filamentous fungi are examined by carefully teasing, with needles, some of the mycelium in a drop of mounting medium, i.e., lactophenol cotton blue or glycerine and eosin, and covering the preparation with a cover glass.

## DERMATOPHYTES

### DEFINITION

The dermatophytes are a closely related group of fungi which cause specific infections of man and animals by invading only the superficial keratinized areas of the body such as the skin, hair and nails. They do not cause systemic infections and rarely, if ever, invade the subcutaneous tissues. In their parasitic habitat, they show a very reduced, rudimentary morphology, appearing only as mycelial fragments in skin and nails or as mycelial fragments and arthrospores arranged inside or outside the hair. In culture on Sabouraud's glucose agar at room temperature, however, they develop filamentous colonies which reproduce by a variety of spore forms characteristic of the group. Three genera are now recognized: *Microsporum*, *Trichophyton* and *Epidermophyton*.

### HISTORY

Schoenlein (1839) reported the first etiologic agent of disease in man when he described a fungus as the cause of favus. Remak (1845) named this fungus *Achorion Schoenleini*. Within a few years, other fungi were reported as the etiologic agents of disease in man. Gruby (1843) described *Microsporum Audouini* and Malmsten (1845) described *Trichophyton tonsurans* as etiologic agents of ringworm of the scalp. Later, Sabouraud (1907) described *Epidermophyton inguinale* from eczema marginatum while Castellani (1910) reported *Endodermophyton concentricum* from tinea imbricata or Tokelau. Complete descriptions of all types of ringworm infection of the hair, skin and nails, as well as the fungi which caused

such lesions, were published by Sabouraud (1910) who listed 45 species of dermatophytes. More than 100 species of *Trichophyton* alone are now listed in the literature. These species were separated not only on the basis of differences in the appearance of lesions from which they were isolated but also of differences observed in the gross appearance of colonies on Sabouraud's standard medium. Critical studies of these dermatophytes, however, have reduced two genera and several species to synonymy with previously described forms (Langeron and Milochevitch, 1930; Ota and Kawatsur , 1933; Emmons, 1934; and Conant, 1941). Thus, the genera *Achorion* and *Endodermophyton* have been discarded and their species have been placed in the genus *Trichophyton*. This genus is now thought to contain only 10 to 12 species while *Microsporum* contains only three species and *Epidermophyton* a single species (Conant et al., 1944).

### CULTIVATION

The dermatophytes may be grown on a variety of simple media but they are usually cultivated on Sabouraud's glucose agar at room temperature because the somewhat typical appearance of the colonies and the microscopic morphology developed on this medium have been used for generic and specific identification. Recent studies of the exact nutritional requirements for some of the dermatophytes may result in a more stable taxonomy of these fungi by allowing better colony formation, more consistent spore production and less variable macroscopic appearances (Robbins and Ma, 1942, 1945; Hazen, 1947).

**Genus *Trichophyton*.** On Sabouraud's glucose agar at room temperature, the colonies are granular to powdery; cottony to velvety; heaped, wrinkled and folded with a velvety surface; or heaped, wrinkled and folded with a smooth and waxy surface. Pigmentation of the colonies varies from delicate pink to red, purple, violet, brown, yellow and light buff.

Microscopically, microconidia are the prominent spore forms. These are subspherical, pyriform or clavate (1.5 to 2 x 2 to 5  $\mu$ ) distributed on the sides of the hyphae (*en thyrses*) or produced on conidiophores in clusters (*en grappe*).

Macroconidia are characteristic but rare and appear as long, thin-walled, multiseptate, clavate spores ( $4 \times 6 \mu$  in width by 10 to  $50 \mu$  in length). Raquette mycelium, nodular bodies, coiled hyphae and chlamydospores are also found in some species.

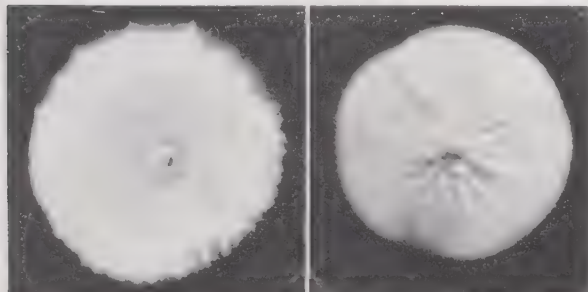


FIG. 39. (Left) *Trichophyton (gypseum) mentagrophytes*, 14 days on Sabouraud's glucose agar at room temperature. (Right) *Trichophyton (interdigitale) mentagrophytes*, 14 days on Sabouraud's glucose agar at room temperature.

TRICHOPHYTON (GYPSEUM) MENTAGROPHYTES (Robin) Blanchard, 1896. Primary cultures may be granular to powdery and light buff to tan in color. Overgrowth of fluffy, cottony, pure white mycelium on transfer produces the "interdigitale" type of colony (Fig.



FIG. 40. Microscopic morphology of *Trichophyton mentagrophytes*. (Left) Conidiophores producing clusters of microconidia (en grappe)  $\times 368$ ; (center) Microconidia borne laterally on hypha (en thyrses)  $\times 368$ ; (right) Macroconidium (fuseau).  $\times 368$ .

39). Microscopically, numerous subspherical microconidia, tightly coiled hyphae, chlamydospores, raquette hyphae and nodular bodies, but few macroconidia, are seen (Fig. 40).

TRICHOPHYTON (PURPUREUM) RUBRUM (Castellani) Sabouraud, 1911. Primary cul-

tures are cottony and pure white but later develop a velvety surface with a rose-purple or reddish pigment on the back of the colony (Fig. 41 top, right). This pigmentation may spread into the agar and into the surface mycelium. Microscopically, numerous clavate microconidia, borne on the sides of the hyphae, chlamydospores and raquette hyphae, but few macroconidia, are seen.

TRICHOPHYTON (CRATERIFORME) TONSURANS Malmsten, 1845. The colony is slow-growing with compact, whitish-cream, velvety

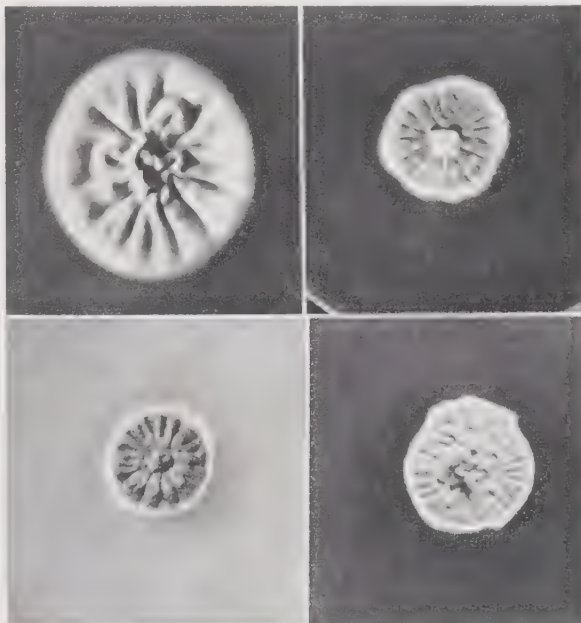


FIG. 41. (Top, left) *Trichophyton tonsurans*, 35 days on Sabouraud's glucose agar at room temperature; (top, right) *Trichophyton rubrum*, 12 days on Sabouraud's glucose agar at room temperature; (bottom, left) *Trichophyton violaceum*, 19 days on Sabouraud's glucose agar at room temperature; (bottom, right) *Trichophyton concentricum*, 12 days on Sabouraud's glucose agar at room temperature.

surface that becomes folded with deep crateriform depressions of yellowish color (Fig. 41 top, left). Microscopically, numerous clavate microconidia, borne on the sides of the hyphae, numerous chlamydospores, hyphal swellings and raquette hyphae, but rare macroconidia, are seen.

TRICHOPHYTON (ACHORION) SCHOENLEINI (Lebert) Langeron and Milochevitch, 1930. The colony is slow-growing, heaped, compact, waxy and smooth with many irregular folds, yellowish-white to light brown in color (Fig.



42 left). On transfer, the smooth, waxy appearance changes to a velvety white. Microscopically, only chlamydospores, hyphal swell-

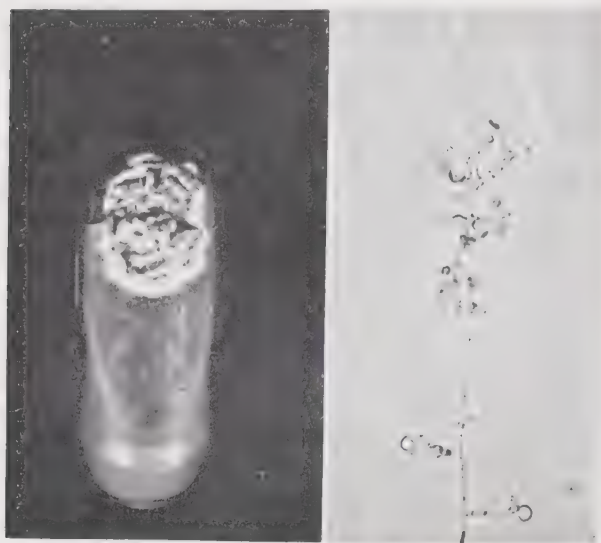


FIG. 42. (Left) *Trichophyton Schoenleini*, 21 days on Sabouraud's glucose agar at room temperature. (Right) Favic chandeliers produced in cultures of *T. Schoenleini*.  $\times 183$ .

ings and the so-called "favic" chandeliers are seen (Fig. 42 right).

**TRICHOPHYTON (ACHORION) VIOLACEUM** Sabouraud, 1902. The colony is slow-growing, heaped, compact, smooth, waxy, with irregular folds and a deep violet pigmentation (Fig. 41

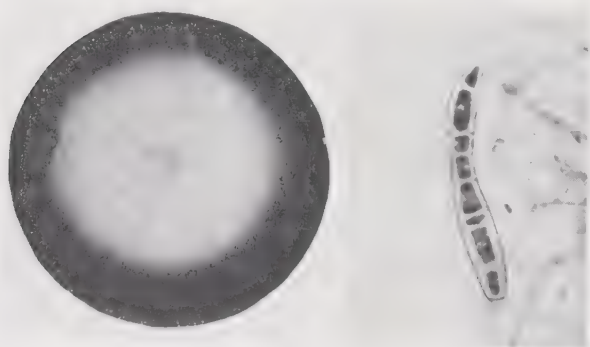


FIG. 43. *Microsporium Audouini*. (Left) Twenty-one days on Sabouraud's glucose agar at room temperature. (Right) Macroconidium (fuseau) elongate, imperfectly formed macroconidia are found in this species,  $\times 351$

bottom, left). Microscopically, only chlamydospores and hyphal swellings with no characteristic spore forms are seen.

**TRICHOPHYTON (ENDODERMOPHYTON) CONCENTRICUM** Blanchard, 1896. The colony is slow-growing, heaped, deeply furrowed, smooth, and brownish in the center (Fig. 41 bottom, right). On transfer, the surface becomes velvety. Microscopically, only chlamydospores, hyphal swellings and the so-called "favic" chandeliers are seen.

**TRICHOPHYTON (MICROSPORUM) FERRUGINEUM** (Ota) Langeron and Milochevitch, 1930. The colony is slow-growing, glabrous, smooth, and orange in color. Microscopically, only chlamydospores and hyphal swellings are seen.

**TRICHOPHYTON DISCOIDES** Sabouraud, 1910. The colony is slow-growing, convex, disklike, moist, glabrous and dull yellow in color. Microscopically, only chlamydospores and hyphal swellings are seen.

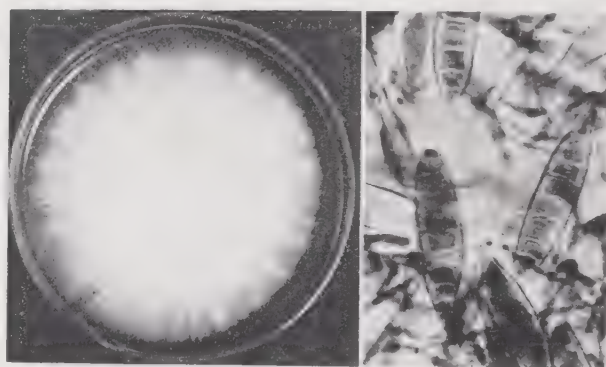


FIG. 44. *Microsporium canis*. (Left) Fourteen days on Sabouraud's glucose agar. (Right) Well developed and numerous macroconidia are found in this species.  $\times 350$ .

**Genus Microsporium.** On Sabouraud's glucose agar at room temperature, the colonies are slow-growing, matted and furrowed, or fast-growing, cottony or powdery, and tan to cinnamon brown in color. The pigmentation in the agar may be reddish-brown to orange. Microscopically, the macroconidia are numerous and characteristic. They are large (8 to 15  $\mu$  in width by 40 to 150  $\mu$  in length), spindle-shaped, multicelled, rough, thick-walled spores. The microconidia (2.5 to 4  $\times$  3 to 6  $\mu$ ), scarce in primary cultures, are borne singly along the hyphae or from short stalks from the hyphae.

**MICROSPORUM AUDOUINI** Gruby, 1843. The colony is slow-growing, matted and velvety, tan to brownish in color with yellowish or orange pigmentation in the agar (Fig. 43 left). Microscopically, the macroconidia are rare and ill-formed in this species (Fig. 43 right). The

microconidia are clavate ( $2.5$  to  $4 \times 3$  to  $6 \mu$ ), borne on the hyphae or from short stalks on the hyphae. Pectinate hyphae, raquette mycelium, chlamydospores and nodular bodies are also seen.



FIG. 45. *Microsporum gypseum*. (Left) Seven days on Sabouraud's glucose agar at room temperature; (right) Macroconidia.  $\times 330$ .

**MICROSPORUM CANIS** Bodin, 1902. The colony is fast-growing with abundant cottony, aerial, buff-tan mycelium and yellowish to orange pigmentation in the agar (Fig. 44 left). Microscopically, numerous characteristic macroconidia are produced. They are large ( $8$  to  $15 \times 40$  to  $150 \mu$ ) multicelled, spindle-shaped, thick-walled spores (Fig. 44 right). Raquette mycelium, chlamydospores and nodular bodies are also seen.

**MICROSPORUM GYPSEUM** (Bodin) Guiart and Grigorakis, 1928. The colony is fast-growing with white, cottony, aerial mycelium which becomes matted and powdery and cinnamon-brown in color (Fig. 45 left). Microscopically, numerous macroconidia are produced. They are elongate and ellipsoid ( $8$  to  $12 \times 30$  to  $50 \mu$ ) multicelled, rounded to tapering at the ends, with rough, thin walls (Fig. 45 right). Raquette mycelium, chlamydospores and nodular bodies are also seen.

**Genus Epidermophyton.** On Sabouraud's glucose agar at room temperature, the colonies are velvety to powdery and greenish yellow in color. Microscopically, only oval to broadly clavate macroconidia are produced. This genus contains one species.

**EPIDERMOPHYTON FLOCCOSUM** (Harz) Langeron and Miloshevitch, 1930. The colony develops with a central, cottony, white aerial mycelium which becomes powdery and greenish-yellow in color (Fig. 46 top). Microscopically, the oval, broadly clavate, two- to six-celled,

smooth, thin-walled macroconidia ( $7$  to  $12 \times 20$  to  $40 \mu$ ) are characteristic for this fungus. They are produced directly from the hyphae or in typical clusters (Fig. 46 bottom). No microconidia are to be found. Older cultures produce many chlamydospores and raquette cells.

#### DISTRIBUTION

The dermatophytes have a worldwide distribution. Some species, however, are found constantly in certain geographic areas and rarely in others.

*T. (Achorion) Schoenleini* is found in the countries bordering the Mediterranean, in the

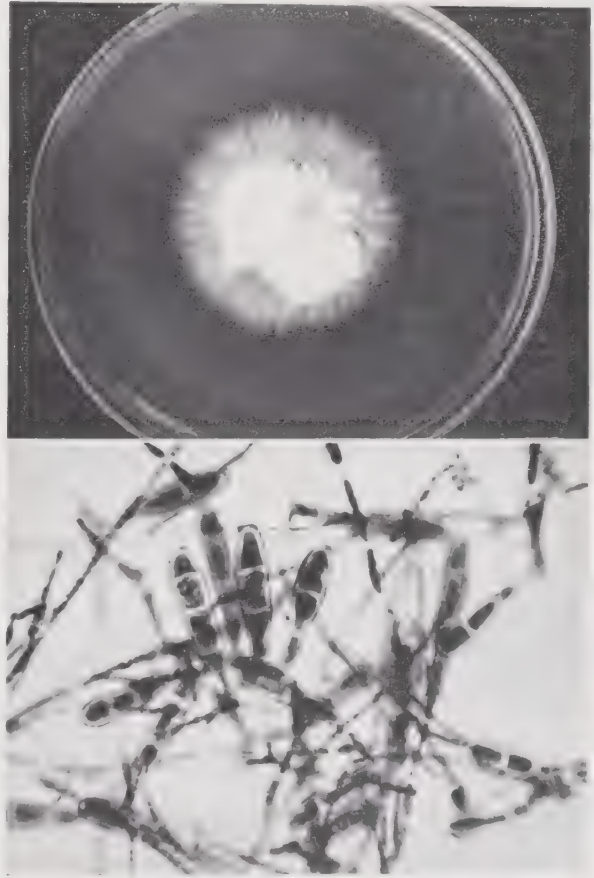


FIG. 46. *Epidermophyton floccosum*. (Top) Twelve days on Sabouraud's glucose agar at room temperature. (Bottom) Typically clustered macroconidia.  $\times 530$ .

Balkans and scattered throughout Europe and the Far East. Cases of infection in this country usually are found in families of recent immigration. *T. (Achorion) violaceum* also is



found in the Balkans, Russia and southern European countries, with scattered cases reported throughout Europe. In the United States, cases of infection by this fungus are sporadic and usually found in foreign families. *T. (Microsporum) ferrugineum* is common in Manchuria and Japan and rarely found elsewhere. *T. (Endodermophyton) concentricum* seems to have a tropical distribution; it has been reported from the Pacific, South America, and occasionally from Central America. No cases have, as yet, been reported from the temperate zone. *T. (purpureum) rubrum* is said to be more prevalent in subtropical areas (Mexico, Central America, West Indies, parts of South America). In the United States, more cases are reported from the southern part of the country.

*Microsporum Audouini* is endemic in Europe (France, Spain, Italy, Germany, Austria and in the Balkans). Until World War I, the occurrence of this fungus was sporadic in England and the United States. During World War II, however, epidemics caused by *M. Audouini* in the United States have caused great concern.

Many of the dermatophytes cause infections in animals and the range of pathogenicity of these fungi is extensive. The cat, dog, horse, calf, cow, sheep, squirrel, monkey, rat, etc., have been found to have spontaneous infections with one or another of the dermatophytes, and laboratory animals such as the guinea pig and rabbit, as well as the cat and dog, may be experimentally infected. Positive blood cultures may be obtained occasionally from guinea pigs with experimentally induced skin lesions, but these animals do not show internal lesions. Intravenous injection of guinea pigs with spore suspensions does not result in infection of the internal organs but specific cutaneous lesions will be formed if the skin of such animals is traumatized. This extreme specialization for, and invasion of, a particular tissue is not duplicated by any of the other pathogenic fungi. Some fungi, such as *M. Audouini*, are considered to be human species in that they are not found on animals and animals cannot be experimentally infected.

## PATHOGENESIS

The dermatophytes cause superficial infections (dermatomycoses) of the keratinized areas of the body, i.e., skin, hair and nails. These fungi do not invade the deeper tissues or internal organs of man and do not cause systemic infections in experimentally inoculated animals.

The most prevalent infection is that referred to as tinea pedis (Athlete's foot, dermatophytosis, etc.) in which the toe webs are invaded by species of *Trichophyton* or *E. floccosum*, resulting in acute, subacute or chronic infections. In most instances, the infection becomes noticeable as a pruritic, vesiculated area between the toes with occasional spread to the rest of the foot. Rupture of the vesicles and discharge of a thin, serous fluid causes maceration and peeling of the tissue. This may be accompanied by the appearance of fissures or cracks. Unless secondary bacterial infection takes place, the lesion usually persists for long periods of time as a macerated area between the toes. Superimposed bacterial invasion, however, may result in an acute inflammatory reaction with lymphangitis or lymphadenitis. Occasionally, certain species of *Trichophyton* cause marked inflammatory reactions and the fungus or its products sensitize the skin. In such cases, vesicular lesions, indistinguishable from primary infections, may appear elsewhere on the body, particularly on the palms of the hands. These lesions are considered to be allergic manifestations or dermatophytids if fungi are not found in them; a primary focus of infection is found elsewhere on the body, and the trichophytin test is positive.

Infection of the nails (tinea unguium) may accompany lesions between the toes or on the feet. Usually only two or three nails are affected and these become discolored, brittle, opaque, lusterless, thickened and friable. Paronychia is not common.

Infection of the glabrous skin of the body (tinea glabrosa) occurs more commonly in

children as a result of contact with infected animals or by autoinoculation with hairs from an infected scalp. Adults may become infected by handling animals or infected children, or from lesions on the nails and feet. Although a variety of lesions on the glabrous skin may be caused by dermatophytes, the typical, annular, ringworm lesion is one with a healing, scaly center and active, erythematous, vesiculopustular border.

Ringworm of the scalp (*tinea capitis*) occurs in childhood and in most instances, if not cured during this period, heals spontaneously at puberty. A few of the dermatophytes, however, cause lesions which tend to hold over into adult life (*T. Schoenleini*, *T. violaceum*, *T. tonsurans*). Infection by *M. Audouini* is acquired by contact with other infected children and usually occurs in epidemics. Infection by *M. (lanosum) canis*, however, is acquired by contact with infected animals (cats and dogs) and is usually sporadic. The appearance of the lesions depends on the infecting fungus, whether a *Microsporum* or *Trichophyton*.

In microsporiasis, the hair is broken off a short distance from the surface of the scalp leaving greyish areas composed of hair stubs surrounded by a sheath of spores. Infection by *M. canis* or *M. gypseum* may also cause an inflammatory reaction resulting in a boggy, tumorlike mass or kerion which resembles a pyoderma. In trichophytosis, species of *Trichophyton* which invade the hair shaft (endothrix) cause small, scattered, scaly lesions with a thinning of the hair where they are broken off at the surface of the scalp leaving follicles with a black center (black-dot ringworm). Another endothrix species, *T. Schoenleini*, causes a characteristic infection of the scalp (favus) characterized by cuplike structures (scutula) formed by the infected hair follicles. Ectothrix species, *T. (gypseum) mentagrophytes*, may produce acute inflammatory reactions resulting in kerion formation.

Infection of the bearded region of man (*tinea barbae*) may be caused by various

species of *Trichophyton* and resemble closely infections due to pyogenic organisms.

Since the dermatophytes invade the horny layer of the epidermis and can live and multiply only in this dead skin, there are few histopathologic changes that are not those of a response to any inflammatory reaction. These fungi cause erythema and edema with inflammation resulting in scaling of the stratum corneum and vesiculation. Microscopically, there is a marked hyperkeratosis, parakeratosis, acanthosis, and dilatation of the vessels of the papillary layer with plasma and cellular infiltration resulting in interstitial edema.

#### IMMUNITY

Children are susceptible to infection of the scalp and body by dermatophytes of human or animal origin but are resistant to infection of the feet. Adults, on the other hand, are susceptible to infection of the feet, nails and glabrous skin but are resistant to infection of the scalp.

Greenbaum (1924) failed to demonstrate circulating antibodies by means of the complement fixation test. Marcussen (1937), by the passive transfer technic (Prausnitz-Küstner), demonstrated circulating antibodies of the urticarial type in individuals with allergic manifestations who gave an immediate wheal to intracutaneous injections of trichophytin. Immunity in humans is usually demonstrated, however, by the cutaneous sensitivity which is established during infection and which can be demonstrated by an intracutaneous injection of trichophytin. The reaction is of the delayed type (24 to 48 hours) and frequently lasts seven days. A positive trichophytin test depends, to some extent, on the type of invading fungus and whether or not an inflammatory reaction is induced. The trichophytin used to elicit the skin test in sensitive individuals contains both group-specific and species-specific antigens (Jadassohn et al., 1937). The trichophytin test may indi-



cate either present or past infection by one of the dermatophytes.

### DIAGNOSIS

Diagnosis is best made by demonstrating the fungus in the hair, skin or nails and by culture. The Wood's light is an invaluable aid in locating and determining the extent of infection on the scalp. Infected hairs fluoresce when this light is held close to the scalp in a semidark room. Hair epilated from lesions on the scalp, skin scraped from the erythematous border of lesions on the

skin or obtained from the roofs of vesicles and scrapings obtained from the discolored, friable areas of infected nails are examined in 10 per cent KOH. Dermatophytes in the skin or nails appear as branching fragments of hyphae (Fig. 47 *top, left*); the genus and species of the invading fungus can be determined only by culture. In the hair the appearance of these fungi, in KOH preparations, allows, to some extent, a generic determination. Species of *Microsporum* form dense spore sheaths around the hair stub with the spores crowded into a mosaic pattern (Fig. 47 *bottom, left*). Species of *Trichophyton* form parallel rows of small or large spores outside the hair shaft (ectothrix-microides; ectothrix-megaspores) or inside the hair shaft (endothrix) (Fig. 47 *top, right* and *bottom, right*). Although the appearance of the infected hair may allow identification of the genus of dermatophyte, the species can be identified only by culture.

Cultures are made by inoculating Sabouraud's glucose agar slants with two to three fragments of infected material. All cultures are maintained for at least two weeks at room temperature.

### TREATMENT

The treatment of scalp infections must be directed toward the epilation of all infected hairs. The hair should be clipped as closely as possible and, with the aid of a Wood's light, the infected areas should be outlined with gentian violet or other suitable dye. Manual epilation of hairs within the infected areas should be attempted 3 or 4 times weekly. The head should be shampooed daily followed by an application of Salicylanilide ointment, copper undecylenate ointment or other topical remedies as suggested by Schwartz, et al. (1946). A clean, cotton, stocking cap (made from the top of a woman's stocking) should be worn at all times and changed daily. Such caps must be boiled for at least ten minutes before washing. Progress of treatment should be

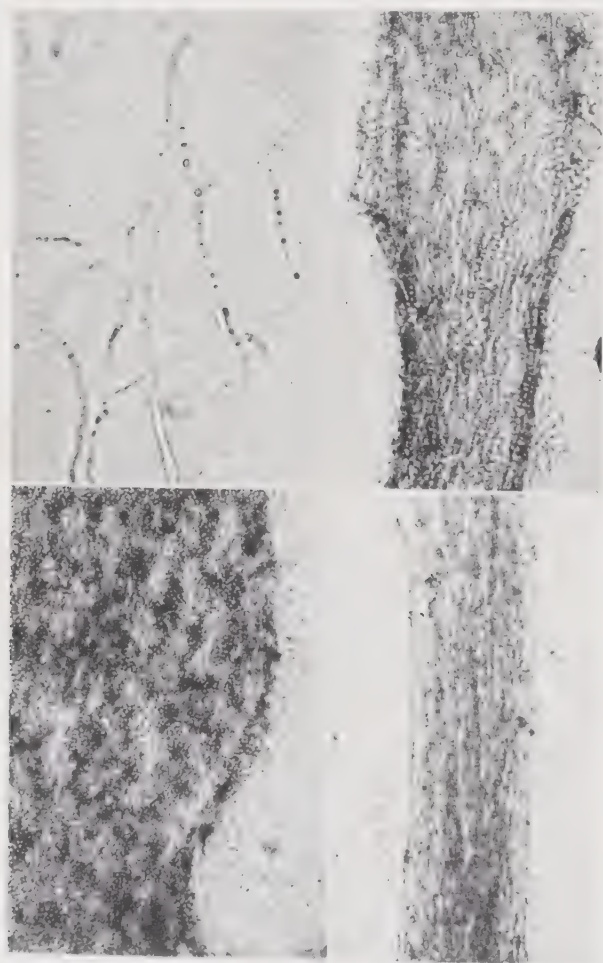


FIG. 47. Potassium hydroxide preparation of skin and hair. (*Top, left*) *Trichophyton mentagrophytes* in skin,  $\times 415$ ; (*top, right*) *Trichophyton* (endothrix) hair,  $\times 170$ ; (*bottom, left*) *Microsporum* hair,  $\times 170$ ; (*bottom, right*) *Trichophyton* (*Achorion*) hair,  $\times 170$ .

followed by weekly examinations under a Wood's light after thorough shampooing of the scalp. Infections caused by *Microsporum (lanosum) canis* should respond in two to three months. Infections caused by *Microsporum Audouini* may respond in three to six months but usually persist until puberty at which time the infection heals spontaneously. Epilation by X-ray, followed by the above routine, is the best method of treatment for infections caused by *M. Audouini*.

Infections of the glabrous skin usually respond readily (two to three weeks) to treatment with tincture of iodine (1 to 3.5 per cent), ammoniated mercury (5 per cent) or a sulfur-salicylic ointment (3 per cent each). Infections due to *T. (purpureum) rubrum* or *T. Schoenleini* demand vigorous treatment over a longer period of time.

Acute infections of the feet should be treated conservatively until all signs of inflammation have disappeared. Continuous compressing with warm boric acid solution, 1:4,000 potassium permanganate solution or physiologic saline should be followed by manual debridement of macerated tissue: all vesicles and bullae should be opened. When the acute process has subsided, a sodium propionate ointment or an undecylenic acid-zinc undecylenate ointment may be applied at night. During the day, a dusting powder may be used (sodium propionate 20 per cent in talc 80 per cent; zinc undecylenate 20 per cent, undecylenic acid 2 per cent in talc 78 per cent). Every effort should be made to avoid overtreatment in fungus infections of the feet.

#### EPIDEMIOLOGY

The dermatophytes include species which primarily infect animals and only incidently infect humans (animal or zoophilic species); and species which infect humans only (human or anthropophilic species). The animal species, e.g., *Microsporum (lanosum) canis*, cause sporadic cases of ringworm of the scalp or glabrous skin of children or the

glabrous skin of adults. Such infections can usually be traced to infected cats or dogs. The human species, *Microsporum Audouini*, causes epidemics of ringworm of the scalp of children by a person to person spread of hairs infected with this fungus. Such hairs are easily dislodged from the scalp and may be picked up from the backs of theater seats, from the clippers in barbershops or by direct contact at play. Since the duration of treatment is long (3 to 6 months) and the incidence of family infections and preschool infections is high, no attempt is made to keep infected children from attending school. Epidemics caused by *M. Audouini* recently have reached such proportions that the disease can be handled best only by Public Health facilities. Steves and Lynch (1947) have reported the trend of these epidemics, and Schwartz et al. (1946) have outlined procedures to follow in such epidemics.

Tinea pedis (Athlete's foot, ringworm of the foot, etc.) is thought to be spread from person to person by the common use of shower baths, etc., in schools, colleges and athletic clubs. The high incidence of infection among the troops during World War II indicated again that communal life and common use of bathing facilities are important factors in spreading this type of infection. Under such conditions, the scuffed or rubbed-off, infected, macerated or peeling skin, from the feet or between the toes, serves as the source of infection.

#### CONTROL MEASURES

Epidemics of tinea capitis in children can be controlled by notifying Public Health authorities of the individual case or cases which may be seen by the private physician. Such officials may then plan to screen all school children by means of the Wood's light and determine the extent of infection in the locality. Preschool children should also be examined when an older member of the family has an infection. Examination and treatment centers may then be estab-



lished by the Public Health authorities at the schools or other convenient places. When stocking caps are worn and treatment instituted as indicated above, the epidemic will be controlled. Specific directions for controlling such epidemics have been published by Schwartz et al. (1946).

Tinea pedis cannot be controlled by the use of foot baths as formerly believed. Such baths (hypochlorite or hyposulfite) are not sufficiently fungistatic or fungicidal, penetration of infected skin is not obtained and the time of immersion is too brief to be of value. Control of infections of the feet should be directed toward individual prophylaxis, adequate treatment and prevention of reinfection. Foot powders (10 per cent boric acid in talc, undecylenic acid and zinc undecylenate in talc, etc.) tend to keep the feet dry and are of value in preventing infection. Sulzberger and Kanof (1947) have recently reported excellent results with such powders in extensive tests. Powders or ointments containing the undecylenic acid and zinc undecylenate were also found to be of value in treatment. Reinfection can be controlled by proper sterilization of previously worn shoes by subjecting the shoes to formaldehyde vapors for 24 hours. Treated shoes should be thoroughly aired before use.

## CRYPTOCOCCUS NEOFORMANS

### DEFINITION

*Cryptococcus neoformans* is a yeastlike, non-sporulating, nonmycelial, budding fungus characterized by the development of a wide capsule both in tissue and in culture. It has a marked predilection for the central nervous system and produces a subacute or chronic infection of the meninges (*Torula meningitis*) or lesions simulating brain tumor or brain abscess, but may also involve the skin, lungs and other organs.

### HISTORY

There are many early reports concerned with budding, yeastlike fungi which, al-

though given a variety of names and isolated from a variety of sources, are considered to be the same fungus: *Cryptococcus neoformans*.

A yeast (*Saccharomyces* sp.) was described in Europe by Busse (1894-1895) from a patient with a localized subperiosteal infection of the tibia, who later died with multiple lesions of the skin and viscera. Curtis (1896) also described a *Saccharomyces*, *S. tumefaciens*, from a myxomatous tumor of the hip. In Italy, Sanfelice (1895) isolated *S. neoformans* from the surface and juice of peaches and compared it with *S. lithogenes* which he isolated from the lymph node of an ox with primary carcinoma of the liver, and found both to be pathogenic for laboratory animals. Later, Sanfelice (1898) described *S. granulomatogenes*, isolated from the lung of swine. Weis (1902) described *Torula Plimmer* from cancer of the breast and *Torula Sanfelice* from an adenocarcinoma of a human ovary. Frothingham (1902) described a *Torula* sp. isolated from a tumor mass in the lung of a horse. In Germany, v. Hansemann (1905) isolated a yeast by lumbar puncture from a case of suspected tuberculous meningitis.

In the United States, Stoddard and Cutler (1916) reported two cases in man presenting signs of cerebral tumor which were caused by a budding fungus. The fungus isolated from one of these cases was compared with that obtained by Frothingham (1902) and was named *Torula histolytica*. All these fungi are now considered to be identical.

### CULTIVATION

*Cryptococcus neoformans* may be cultured at room temperature or at 37° C. on all common laboratory media. On Sabouraud's glucose agar at room temperature, the colony is glistening, mucoid, and tan to brown in color (Fig. 48). Microscopically, the cultures are best examined by emulsifying a portion of the growth in a drop of water or India ink under a cover glass. Such preparations reveal thick-walled, ovoid to spherical, budding cells.

5 to 15  $\mu$  in diameter (Fig. 49), surrounded by a wide, gelatinous capsule; no endospores are produced, and no mycelium is developed. Some strains have been reported which produce acid in a few carbohydrates, especially glucose, but the fungus is generally considered to exhibit little biochemical activity.

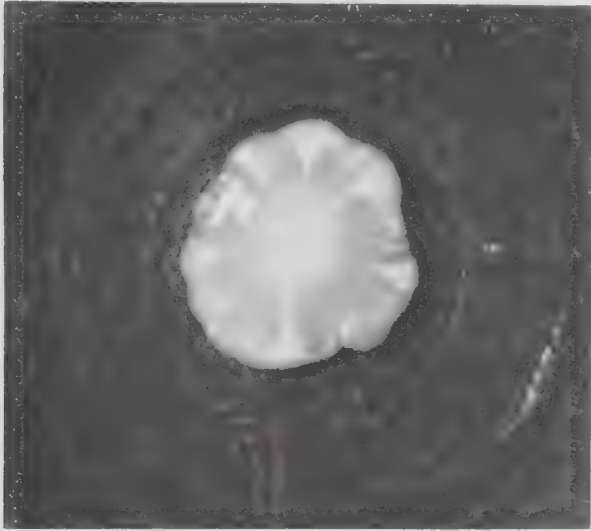


FIG. 48. *Cryptococcus neoformans*, 12 days on Sabouraud's glucose agar at room temperature.

#### DISTRIBUTION

Human infections have been reported from Europe, India, Australia, Japan, Honduras, South America, South Africa, Canada and the United States. In the United States, most of the cases have been reported from the southern part of the country. *Cryptococcus neoformans* is pathogenic for rats and mice and can be distinguished from similar but nonpathogenic cryptococci isolated from the skin and feces (Benham, 1935) by a virulence test in these animals. As previously mentioned, various animals are also subject to infection: ox, swine, horse (Frothingham, 1902; Harrison, 1928) and cheetah (Weidman and Ratcliffe, 1934).

#### PATHOGENESIS

Cryptococcosis is a subacute or chronic infection caused by *C. neoformans* which may affect the skin, lungs or other tissues

of the body with almost invariable meningeal involvement terminally. Clinically, cutaneous and systemic types of infection have been described. The cutaneous may be primary or appear as a manifestation of an already established systemic infection. Cutaneous lesions may appear as acneform pustules, punched-out granulomatous ulcers, subcutaneous tumors or deep-seated abscesses. Many cutaneous cases progress to generalized infection with involvement of the lungs, visceral organs and the central nervous system. Systemic cryptococcosis may involve the brain, meninges, lungs, liver, spleen, pancreas, thyroid and aorta. In the majority of cases the central nervous system is the most frequently involved, the lungs occasionally and other organs seldom. Primary pulmonary infections resemble neoplasm or tuberculosis. Brain infection may resemble an encephalitis, acute or chronic meningitis of bacterial origin (especially tuberculous meningitis), brain tumor, brain abscess, central nervous system degenera-



FIG. 49. *Cryptococcus neoformans*, round, yeastlike cells with slight halo from Sabouraud's glucose agar at room temperature.  $\times 490$ .

tion or central nervous system syphilis. The spinal fluid pressure is increased, globulin and albumin increased, cell count high, chlorides and sugar content low.

The histology of the lesions in the brain varies greatly: some sections show only a



minimal reaction with surprising lack of inflammatory cells; other sections may show pseudotubercles formed of giant cells, epithelioid cells and lymphocytes. The centers of such lesions may be necrotic or hyalinized.

#### IMMUNITY

Most attempts to demonstrate antibody response by the patient to *Cryptococcus neoformans* have produced negative results.

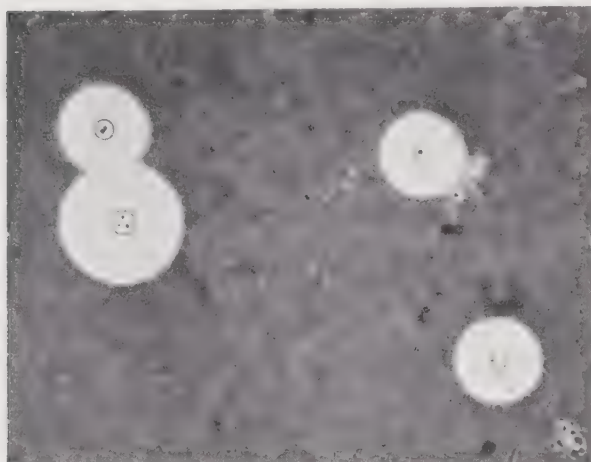


FIG. 50. *Cryptococcus neoformans*, India-ink preparation of spinal fluid.  $\times 560$ .

Benham (1935) has shown that the encapsulated fungus does not cause antibody formation when injected into animals. Good titers were obtained by injecting the organisms only after the capsular material had been removed. It is conceivable that the capsule may protect the fungus during the disease process.

#### DIAGNOSIS

Sputum, pus, gelatinous exudates or sediment of centrifuged spinal fluid should be examined unstained by placing a small amount of the material on a slide and gently pressing to a thin film under a cover glass. Also, these materials should be mixed with a small amount of India ink and examined under a cover glass before the preparation dries. The fungus appears as a thick-

walled, spherical, budding, yeastlike cell, 5 to 15  $\mu$  in diameter, surrounded by a wide capsule (Fig. 50). Specimens should be cultured on Sabouraud's glucose agar at room temperature and blood agar at 37° C.

Some infected materials, particularly spinal fluid, may also be injected intraperitoneally and/or intracerebrally into mice to recover the fungus. Also, a saline suspension of the culture should be injected into mice to distinguish the pathogenic *Cryptococcus neoformans* from some of the nonvirulent but morphologically similar cryptococci (Benham, 1935).

#### TREATMENT

Cutaneous cryptococcosis usually develops into the generalized form of infection in which prognosis is poor. Occasional cases have been successfully treated (Kessel and Holtzworth, 1935; Dienst, 1938) with iodides, X-ray, excision of small infected areas or amputation. Systemic cryptococcosis is almost invariably fatal. Reports concerning the use of sulfonamides and penicillin suggest excellent results in some cases and poor results in others. Reeves, Butt and Hammack (1941) reported successful treatment with sulfapyridine while Marshall and Teed (1942) found sulfadiazine to be effective. On the other hand, Jones and Klinck (1945) found both sodium sulfadiazine and penicillin to be ineffective in a case of meningitis as well as with *in vitro* experiments with cultures and *in vivo* experiments with mice.

#### EPIDEMIOLOGY

*Cryptococcus neoformans* has been isolated from materials in nature and has been reported from spontaneous infections in animals. No known cases of cross infection from animals to man have been reported, nor has it been possible to establish a natural source of infection. It is not known with certainty whether infection takes place

through the lungs, skin or intestinal tract. Evidence would seem to point to the lungs where healed, tuberculouslike lesions have been found at autopsy, although such lesions, if caused by the *Cryptococcus*, cannot be distinguished from healed tuberculous lesions unless the fungus can be demonstrated.

## CANDIDA ALBICANS

### DEFINITION

*Candida albicans* is an oval, budding, yeastlike fungus producing both blastospores and pseudomycelium in tissue and exudates, and in culture at room temperature and at 37° C. Its exact etiologic significance in any disease process is difficult to establish since it may frequently be found in the normal mouth and intestinal tract or as a secondary contaminant in other recognized diseases.

### HISTORY

Langenbeck (1839) demonstrated, in thrush, the presence of a yeastlike, budding fungus which Robin (1853) named *Oidium albicans*. Zopf (1890) renamed this fungus *Monilia albicans*. The rudimentary morphology of *M. albicans* and the frequency with which similar yeastlike organisms have been isolated from infected and contaminated materials has made classification and identification of this group difficult (Conant, 1940). In the past few years, however, the single genus *Candida* (Berkhout, 1923) has been proposed for these yeastlike fungi and the number of species has been greatly reduced (Benham, 1931; Martin et al., 1937; Langeron and Guerra, 1938; Martin and Jones, 1940; Diddens and Lodder, 1942; MacKinnon and Arttagaveytia-Allende, 1945). *Candida albicans*, with few exceptions, is considered to be the only pathogenic member of the genus. It has been isolated from diseases of the skin, nails, mouth, lungs and vagina and from systemic moniliasis.

### CULTIVATION

*Candida albicans* may be cultured on all common laboratory media both at room temperature and at 37° C. On Sabouraud's glucose agar at room temperature, the colonies are cream colored and soft and have a distinct yeastlike odor (Fig. 51). The surface growth is composed of oval, budding cells, 2.5 x 4 to 6  $\mu$ , while the submerged growth is composed of pseudomycelium. This pseudomycelium, in



FIG. 51. *Candida albicans*, 20 days on Sabouraud's glucose agar at room temperature.

slide culture preparations, is seen to consist of elongate, undetached cells with clusters of blastospores distributed at the points of constriction. On corn meal agar, typical chlamydospores are produced (Fig. 52). There is no surface growth on Sabouraud's glucose broth; glucose and maltose are fermented with acid and gas, sucrose with acid only and lactose is not affected.

*C. albicans* can be differentiated from other commonly isolated yeastlike fungi by the technic of Martin, et al. (1937) and Martin and Jones (1940). The different species of *Candida* are presented in Table 50.

### DISTRIBUTION

*Candida* species are inhabitants of the normal mouth, intestinal tract and vagina and may be cultured from these locations in 35 to 40 per cent of normal individuals. Of this number, about 15 to 20 per cent



TABLE 50. DIFFERENTIAL DIAGNOSIS OF SPECIES OF *Candida* \*

		NONPATHOGENIC						
PATHOGENIC		<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. pseudotropicalis</i>	<i>C. Krusei</i>	<i>C. parakrusei</i>	<i>C. stellatoidea</i>	<i>C. Guilliermondii</i>
Sabouraud's agar		Creamy growth	Not characteristic	Not characteristic	Flat, dry	Creamy	Creamy	Creamy growth
Sabouraud's broth		No surface growth	Narrow surface film with bubbles	No surface growth	Wide surface film	No surface growth	No surface growth	No surface growth
Blood agar.....		Medium-sized, dull-gray colonies	Large, gray colonies surrounded by mycelial fringe	Colonies small, not characteristic	Colonies small, irregularly shaped, flat or heaped	Colonies small, brilliant white	Colonies star-shaped	Medium-sized, dull-gray colonies
Corn meal agar		Branched, treelike mycelium with chlamydospores	Mycelium well developed, branched, bearing numerous blastospores, no chlamydospores	Mycelium poorly developed, no chlamydospores	"Crossed sticks" mycelium, no chlamydospores	Mycelium well developed, no chlamydospores	Mycelium with large, ball-like clusters of blastospores	Mycelium well developed, no chlamydospores
Glucose	AG		AG	AG	AG	AG <sup>1</sup>	AG	2
Maltose	AG		AG	AG			AG	
Sucrose	A		AG	AG				
Lactose.....								

<sup>1</sup> Occasionally acid only.

<sup>2</sup> Langeron and Guerra report acid and gas produced in glucose and sucrose when cultured at 25° C. and held twenty days.

\* Martin, D. S., Jones, C. P., Yao, K. F., and Lee, L. E., Jr., 1937, A practical classification of the monilias. Journal of Bacteriology, 34, 99-129.

have been identified as *C. albicans*. While a few species of yeastlike fungi can be isolated from normal skin, *C. albicans* has not been cultured from skin except when associated with a cutaneous lesion. *C. albicans* is frequently found in the sputum of patients with proven nonmycotic, pulmonary

vaginitis); infections of the skin, particularly of the intertriginous areas (axillae, inframammary, inguinal, intergluteal, interdigital webs of the hands and feet); infections of the nails (onychia and paronychia); and systemic infections (bronchopulmonary, or generalized infection of lungs, lymph nodes, liver, spleen and meninges).

Infection of the mouth (thrush) is encountered more frequently in children than in adults. In children it usually occurs as a result of infection during birth from a mother with a vaginal infection. In adults, infection with *C. albicans* usually follows a debilitating illness. Such lesions appear as extensive or scattered whitish patches which contain the blastospores and pseudomycelium of the fungus. Chronic oral lesions may last several years (Robinson and Tasker, 1947). Occasionally, the fungus spreads to the skin and gastro-intestinal tract to produce a generalized, fatal moniliasis.

Vulvovaginitis, caused by infection of the vaginal mucosa and vulva, is a thrushlike infection characterized by irritation, pruritus and a thin discharge.

Infection of the skin usually occurs by autoinoculation of *C. albicans* from the mouth or intestinal tract. Intertriginous lesions of the hand follow maceration of tissue by continued immersion in water. Such lesions occur frequently in housewives, waiters, chefs, bartenders, fruit canners, etc. Intertrigo of the axillae and intergluteal folds may become established because of obesity or diabetes. Other intertriginous areas showing occasional infection are inframammary folds, groin and interdigital webs of the toes. Such lesions are characterized by erythematous, exudative areas with well-defined vesicopustular or papulosquamous borders.

Infection of the nails (onychia and paronychia) is characterized by swelling at the nail bed which may be painful and resemble a pyogenic infection, and by thickened, transversely grooved nails.



FIG. 52. *Candida albicans*, pseudomycelium with characteristic chlamydospores from corn meal agar culture.  $\times 653$ .

disease (tuberculosis and carcinoma). It is also found in quantity in the stools of patients with diarrheal symptoms due to other causes (sprue and pernicious anemia).

Of the several yeastlike fungi of the genus *Candida*, only *C. albicans* is pathogenic for laboratory animals. Rabbits injected intravenously with 1 cubic centimeter of a 1 per cent saline suspension die in 4 to 5 days with typical abscesses in the kidneys.

#### PATHOGENESIS

*Candida albicans* may cause infections of the mucous membranes of the mouth (thrush) and vagina (vaginitis or vulvo-



Infections of the lungs may cause a mild bronchopulmonary moniliasis with persistent cough, and with sputum containing cellular debris and yeastlike cells. X-rays reveal slight to moderate peribronchial thickening and scattered rales may be heard at the base of the lungs. More extensive pulmonary moniliasis may resemble miliary tuberculosis with cough, fever, dyspnea, chest pain, hemoptysis and night sweats accompanied by signs of pleural thickening and consolidation.

Systemic infection usually follows chronic, refractory skin and oral lesions (Rockwood and Greenwood, 1934). Infrequently, such infections may lead to meningitis (Smith and Sano, 1933; Miale, 1943).

Histologically, sections of skin may show abscess formation or a chronic inflammatory reaction with giant cell formation. Routine staining (H and E) may reveal tuberculoid granulomata with giant and epithelioid cells characteristic of tuberculosis. When Gram stained, however, such sections reveal the Gram-positive blastospores and hyphal segments of *C. albicans* in the centers of the tuberclelike structures and in areas of necrosis.

Moniliids occasionally accompany localized infections. Such lesions are sterile and appear on the body as a result of sensitivity to the yeastlike fungi found in lesions elsewhere on the body.

Although *C. albicans* is considered to be the only truly pathogenic member of the genus, five cases of mycotic endocarditis have been reported (Joachim and Polayes, 1940; Wikler, et al., 1942) from which *C. parakrusei* was cultured from four and *C. Guilliermondi* from one.

#### IMMUNITY

The sera of patients with moniliasis will frequently show agglutination with saline suspensions of *C. albicans*. About 40 or 50 per cent of adults show a positive skin test to *C. albicans* vaccine or oidiomycin. Both

these tests have doubtful diagnostic significance. The constant finding of *Candida* species on the skin, in the mouth and in the intestinal tract of apparently normal individuals could account for both agglutinins and sensitivity to these fungi. It has been shown (Drake, 1945) that, by a slide agglutination technic, 45 per cent of normal human sera agglutinate *C. albicans*. These findings indicate that agglutination tests have little value. Hypersensitivity, however, should be considered and properly evaluated when treating a case of moniliasis.

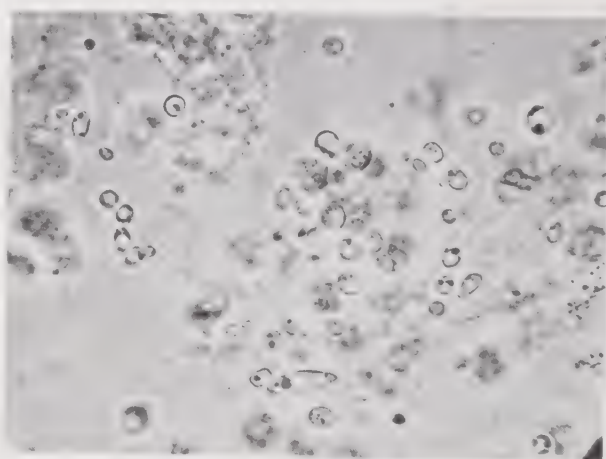


FIG. 53. *Candida albicans*, budding, yeastlike cells in fresh preparation of sputum.  $\times 620$ .

#### DIAGNOSIS

Sputum and materials from lesions in the mouth and in the vagina should be examined as fresh cover glass preparations and as Gram-stained smears. Skin or nail scrapings should be placed in a drop of 10 per cent KOH under a cover glass and the preparation gently heated. In fresh preparations, *C. albicans* appears as an oval, budding, yeastlike fungus,  $2.5 \times 4$  to  $6 \mu$ , with occasional hyphal fragments,  $2.5 \times 6$  to  $12 \mu$  (Fig. 53). In Gram-stained smears, *C. albicans* appears as Gram-positive, oval, budding, yeastlike cells and Gram-positive elongated hyphal cells.

All materials should be cultured on Sabouraud's glucose agar at room temperature and at  $37^{\circ}\text{C}$ . Cultures will become ap-

parent in 4 or 5 days at room temperature, or within 24 to 48 hours at 37° C.

### TREATMENT

Oral and vaginal moniliasis may respond to alkaline mouth washes or alkaline douches, respectively. In both cases, a 1 per cent gentian violet (in 10 to 20 per cent alcohol) used twice daily as a paint for 4 or 5 days has proved effective. Recently, a propionate vaginal jelly (propion gel) has been reported as giving excellent results in vulvovaginitis (Alter et al., 1947). Chronic oral or vaginal moniliasis, however, may resist all therapy including desensitization with oidiomycin.

Intertriginous areas (hands or feet) should be treated with potassium permanganate soaks (1:4,000) three times daily followed by a 1 per cent gentian violet (in 10 to 20 per cent alcohol) paint or a 5 per cent ammoniated mercury ointment. Other cutaneous areas may be treated similarly or with other materials [tincture of iodine, salicylic-benzoic acid ointment, chrysarobin (5 to 10 per cent), etc.]. Onychia and paronychia may fail to respond to any of the above procedures. Fractional X-ray therapy may prove successful.

Bronchopulmonary moniliasis is best treated with potassium iodide by mouth. In a few cases, ethyl iodide inhalation has been used successfully. In the presence of hypersensitivity, the patient should be desensitized with *C. albicans* vaccine before iodides are administered (Conant et al., 1944). Sodium iodide or gentian violet may be given intravenously in certain cases of pulmonary moniliasis. Serum therapy (immune rabbit serum) of a case of widespread pulmonary moniliasis proved successful in a patient who had a negative skin test to *C. albicans* vaccine, a negative agglutination reaction to *C. albicans* antigen but showed a positive Foshay type of reaction to an injection of immune serum previous to serum therapy (Hiatt and Martin, 1946).

Serum therapy, however, can be expected to prove successful only in those cases showing an excess of antigen and an absence of circulating antibody as demonstrated by a negative skin test and a negative agglutination reaction.

### EPIDEMIOLOGY

Yeastlike fungi are found in the mouth, in the intestinal tract, in the vagina and on the skin of normal individuals. The presence of a high percentage (40 to 50 per cent) of positive skin reactions to oidiomycin or to an autogenous vaccine indicates that individuals may become hypersensitive to these organisms or their products. Auto-inoculation from any of the sites mentioned can cause clinical infection.

### CONTROL MEASURES

Patients with clinical moniliasis should be examined and treated for the presence of yeastlike fungi in the mouth or intestinal tract or other obviously infected areas.

## BLASTOMYCES DERMATITIDIS

### DEFINITION

*Blastomyces dermatitidis* is a spherical, thick-walled, budding, yeastlike fungus in tissue or exudates and in culture at 37° C. In culture at room temperature, it develops slowly as a typical, moldlike, filamentous fungus. It produces a granulomatous infection of the skin and internal organs very similar clinically and histologically to tuberculosis. This disease is usually referred to as North American blastomycosis or Gilchrist's disease.

### HISTORY

Gilchrist (1896) first described blastomycetic dermatitis, a disease resembling tuberculosis, from biopsy specimens which showed refractive, double-contoured, budding cells in section. A second case was reported by Gilchrist and Stokes (1896)



from which they obtained a culture of the fungus and named it *Blastomyces dermatitidis* (Gilchrist and Stokes, 1898). Since these initial reports, several fungi have been described as having been isolated from

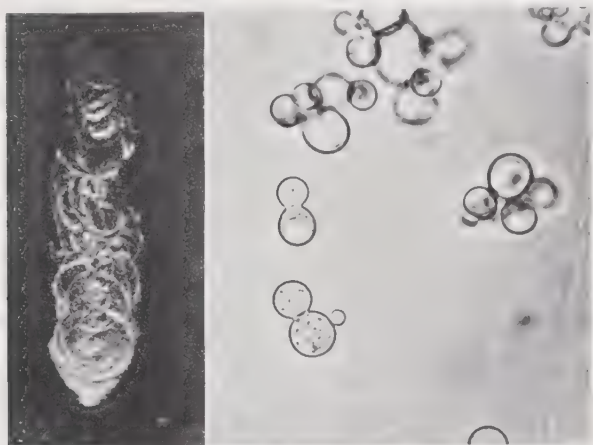


FIG. 54. *Blastomyces dermatitidis*. (Left) Yeastlike culture on blood agar seven days at 37° C. (Right) Yeastlike, single budding cells from blood agar culture.  $\times 587$ .

North American blastomycosis, namely, *Glenospora Gammeli*, *Blastomycoides tulanensis*, *Monosporium tulanense*, *Endomyces capsulatus*, *Endomyces dermatitidis*, and *Glenospora brevis*. A comparative study of these fungi (Benham, 1934; Conant, 1939) has shown them to be identical with Gilchrist's fungus, *Blastomyces dermatitidis*.

#### CULTIVATION

*Blastomyces dermatitidis* may be grown at room temperature or 37° C. on all common laboratory media. On blood agar or beef infusion glucose agar at 37° C., the culture becomes wrinkled, waxy, and yeastlike in consistency (Fig. 54 left). Microscopically it is composed of short, broad, 3- to 4-celled hyphal segments and budding, yeastlike cells, 8 to 20  $\mu$  in diameter, similar to those seen in exudates or sections (Fig. 54 right). On Sabouraud's glucose agar at room temperature, the growth is at first smooth and yeastlike but quickly develops aerial projections and becomes prickly. At this time, a few budding

cells may be found in such cultures but the majority of the growth has become filamentous. A final overgrowth by white aerial mycelium, which may turn brown with age, establishes the completely filamentous stage of the fungus (Fig. 55). Microscopically, such a culture shows spherical to pyriform spores, 5 to 8  $\mu$  in diameter, attached directly to the hyphae or at the ends of short pedicels (Fig. 56). This filamentous form can be converted to the yeastlike, tissue phase by subculturing to blood agar and incubating at 37° C.

Gelatin is not liquefied; sugars are not fermented, nor is milk affected.

#### DISTRIBUTION

So far as is known, *Blastomyces dermatitidis* is confined to the United States and Canada. The incidence of blastomycosis in any area seems to be related directly to an interest shown in the disease and an effort to find cases. Two cases of spontaneous

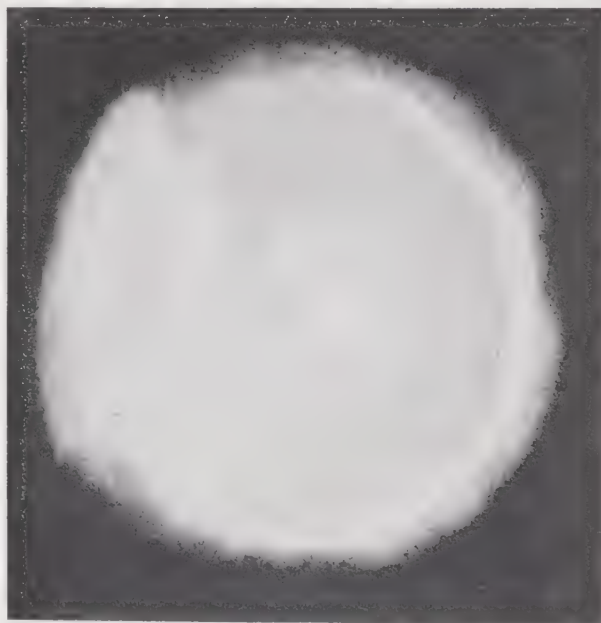


FIG. 55. *Blastomyces dermatitidis*, 21 days on Sabouraud's glucose agar at room temperature.

infection in dogs have been reported: one by Martin and Smith (1936) and the other by Foshay and Madden (1942). Mice, guinea pigs, rabbits and monkeys can be experimentally infected.

## PATHOGENESIS

*Blastomyces dermatitidis* causes a chronic granulomatous infection of the skin and internal organs. Infection usually starts as a papulopustule which spreads peripherally, showing a granulating base covered with a dirty pink exudate and a raised papilliform or verrucous border with miliary abscesses. Spontaneous healing of the center of such

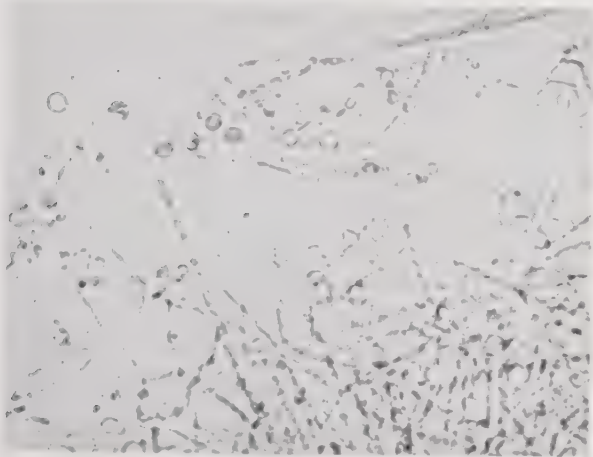


FIG. 56. *Blastomyces dermatitidis*, filamentous stage with conidia from Sabouraud's glucose agar culture at room temperature.  $\times 490$ .

lesions produces scars of tissue paper thinness surrounded by the characteristic raised and spreading border. The cutaneous lesions appear on the exposed parts of the body: face and neck; dorsum of hands, wrists and forearms; ankles and forelegs. Occasional lesions seen in the genitorectal region resemble granuloma inguinale.

In systemic blastomycosis the lungs are most frequently infected and show the most extensive lesions. With hematogenous spread, the skin, subcutaneous tissues and bones are most commonly affected. Skin lesions appear anywhere on the body as multiple, subcutaneous, gummalike lesions which rupture spontaneously freeing bloody pus. The vertebrae and ribs are the bones most frequently affected.

Lesions are also found in the central

nervous system; liver, spleen and kidneys, where they are minimal, and in the prostate. The intestines are not affected.

Histologically the lesions consist of numerous miliary abscesses containing polymorphonuclear cells, cellular debris and giant cells. Also, tuberclelike lesions may be found which are indistinguishable from those seen in tuberculosis unless the fungus can be demonstrated.

## IMMUNITY

Complement fixing antibodies can be demonstrated in the serum of patients with extensive or progressive infection. It is thought, however, that these antibodies denote extent of infection and signify a poor prognosis, since they cannot be demonstrated in patients with localized cutaneous lesions. Hypersensitivity to *Blastomyces* vaccine can be demonstrated in most cases; a delayed tuberculinlike reaction becomes positive in 24 to 48 hours.

## DIAGNOSIS

Crusts from verrucous lesions should be placed in a drop of 10 per cent KOH under a cover glass and examined microscopically after the preparation has been gently heated. Sputum, and pus from miliary abscesses at the border of cutaneous lesions or from subcutaneous lesions, should be examined under a cover glass as untreated, fresh preparations. Spinal fluid and urine should be centrifuged and the sediment examined. In all of these materials, *Blastomyces dermatitidis* appears as a thick-walled, single budding, yeastlike fungus, 8 to 20  $\mu$  in diameter (Fig. 57).

Culture materials on blood agar at 37° C. and on Sabouraud's glucose agar at room temperature. Heaped, wrinkled, isolated colonies which appear on blood agar should be transferred to slants for further development and identification.



## TREATMENT

The sulfonamides and penicillin seem to have no effect on the course of this disease. Cutaneous lesions are best treated with iodides by mouth and X-ray directed at the spreading border of the lesion. Patients with systemic blastomycosis should receive supportive treatment: a high-caloric, high-vitamin diet, with continued bed rest. Io-

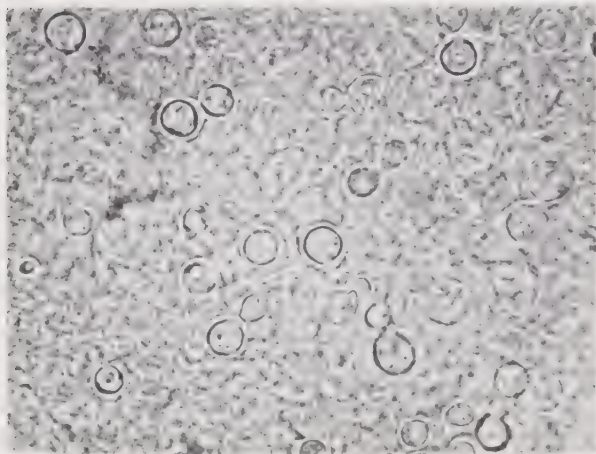


FIG. 57. *Blastomyces dermatitidis*, yeastlike, single budding cells in pus.  $\times 475$ . (Conant, N. F., Martin, D. S., Smith, D. T., Baker, R. D., and Callaway, J. L., 1944, *Manual of Clinical Mycology*. Philadelphia, Saunders, p. 36.)

ides by mouth have been given with varying degrees of success. In both the cutaneous and systemic infections, however, iodides should not be given until the patient has been tested for hypersensitivity to the fungus (Martin and Smith, 1939). The patient should receive desensitizing injections of *Blastomyces* vaccine before iodides are started.

## EPIDEMIOLOGY

*Blastomyces dermatitidis* probably exists in nature but it has never been isolated from natural substrates. Since the localized cutaneous lesions are found on exposed parts of the body, it is assumed that infection follows trauma (Toepel, 1929; Mc-

Kenty and Morgan, 1915; Robinson, 1931). Although systemic blastomycosis is essentially a respiratory infection, there seems to be no person-to-person transmission of the disease.

## BLASTOMYCES BRASILIENSIS

## DEFINITION

*Blastomyces brasiliensis* is a large, thick-walled, single and multiple budding, yeastlike fungus in exudates and tissues, and in culture at  $37^{\circ}\text{C}$ . In culture at room temperature, the fungus develops slowly as a heaped, cerebriform, glabrous, smooth colony or as a slow-growing colony with a short, white aerial mycelium. It produces a granulomatous infection of the mucous membranes of the mouth, lymph nodes and internal organs. The disease is known as South American blastomycosis, Paracoccidioidal granuloma or Lutz-Splendore-Almeida's disease.

## HISTORY

Lutz (1908) first reported pseudococcidioidal granuloma, a localized disease of the mouth and regional lymphatics occurring in Brazil, in which he described an organism thought to be closely related to *Coccidioides immitis*. Carini (1908) reported a second case of localized buccal infection and called the disease blastomycosis. The first generalized infection was described as blastomycosis by Splendore (1909) who later (1912) named the organism *Zymonema brasiliense*. Haberfeld (1919) renamed this fungus *Zymonema histosporocellularis* while Arantes (1922) and Fonseca (1928-1929) described it as *Coccidioides immitis*. Almeida (1930), however, compared cultures of *C. immitis* with cultures from South American blastomycosis and found them to be different. He named the South American fungus *Paracoccidioides brasiliensis*, and Moore (1935-1938) added two new species, *P. cerebriformis* and *P. tenuis*. Conant and Howell (1942) reduced these species to synonymy with *P. brasiliensis* and placed

the South American fungus in the genus *Blastomyces* as *B. brasiliensis*.

#### CULTIVATION

*Blastomyces brasiliensis* can be grown at room temperature or 37° C. on all common

broad, thick-walled cells, 2 to 3 x 4  $\mu$  in size, which easily dissociate. Numerous intercalary and terminal chlamydospores and many non-characteristic hyphal swellings are seen in the mycelium. On the aerial mycelium, some strains develop round to pyriform, sessile conidia, 3 to 5  $\mu$  in diameter.

#### DISTRIBUTION

*Blastomyces brasiliensis* is confined to South America: 750 cases have been reported from Brazil (de Almeida et al., 1946). Isolated cases have been seen in Argentina, Paraguay, Peru and Venezuela. There have been no reports of spontaneous infections in animals. Mice can be inoculated intraperitoneally, and guinea pigs intraperitoneally or intratesticularly.

#### PATHOGENESIS

*Blastomyces brasiliensis* causes a chronic granulomatous infection of the mucous membranes of the mouth and the adjacent



FIG. 58. *Blastomyces brasiliensis*. (Left) Yeastlike culture, 12 days on beef infusion glucose agar at 37° C. (Right) Multiple budding cells and moniliform cells from yeastlike culture at 37° C.  $\times 450$ .

laboratory media. On blood agar or beef infusion glucose agar at 37° C., the culture becomes smooth, waxy and yeastlike (Fig. 58 left). It is composed of numerous round, multiple-budding, yeastlike cells, 6 to 30  $\mu$  in diameter, with buds 1.5 to 5  $\mu$  in diameter scattered over the surface of the parent cell (Fig. 58 right). Frequently, single-budding cells, 8 to 14  $\mu$  in diameter, are also seen. These cells are identical with the yeastlike single-budding forms seen in *Blastomyces dermatitidis*. There can also be seen short, 2- to 4-celled, moniliform chains from the cells of which multiple buds are produced (Fig. 58 right). On Sabouraud's glucose agar at room temperature, *B. brasiliensis* grows slowly (1.5 to 2 cm. in diameter in three weeks) forming a compact culture which may be smooth at first but later develops short, aerial mycelium, white to light brown in color (Fig. 59). These cultures are composed of hyphae with short,

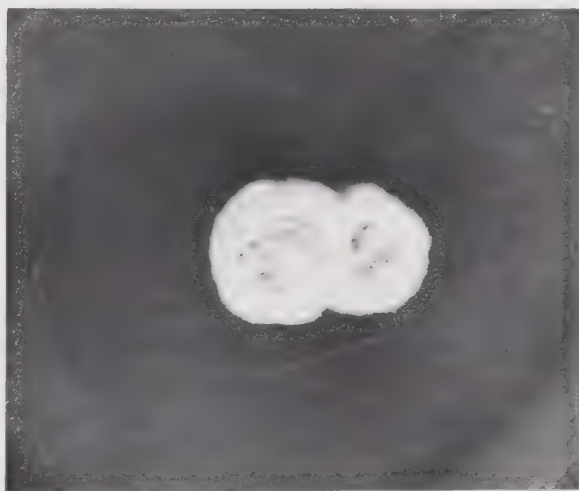


FIG. 59. *Blastomyces brasiliensis*, filamentous colony, 28 days on Sabouraud's glucose agar at room temperature.

skin of the face, the lymph nodes and the viscera. The mouth seems to be the portal of entry where ulcerating, vegetative papillomatous lesions on the buccal mucosa spread to the adjacent skin of the lips and nose. These lesions resemble those of yaws



or mucocutaneous leishmaniasis. The infection may spread to the regional lymphatics and hence to the axillary, inguinal and other nodes. Occasionally, direct infection of the lymph glands of the neck, without demonstrable buccal lesions, produces massive glandular enlargement similar to that seen in Hodgkin's disease. In the visceral type of infection, the intestines serve as the portal of entry. Infection of the

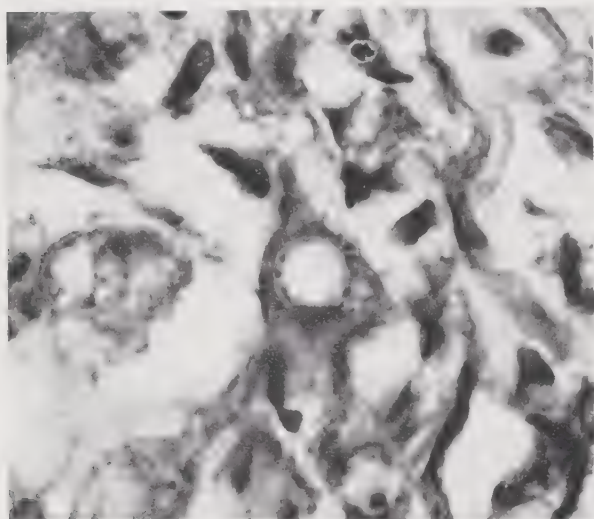


FIG. 60. *Blastomyces brasiliensis*, section of liver showing large, thick-walled cells with minute, coccuslike buds.  $\times 1075$ .

lymphoid tissue of the intestine with lymphatic drainage to the mesenteric nodes, spleen and liver serves to establish massive visceral infection. The lungs are frequently not affected.

Histologically, many areas show abscess formation with predominant polymorphonuclear infiltration; other areas show focal lesions with necrotic and caseous centers surrounded by macrophages, lymphocytes, giant cells and fibroblasts. The organisms appear in the tissue or giant cells as large, round (10 to 60  $\mu$  in diameter) cells with small (1 to 5  $\mu$ ) or large (10 to 30  $\mu$ ) peripheral buds (Fig. 60). This multiple budding is characteristic for *B. brasiliensis*. Frequent single budding forms, 10 to 20  $\mu$  in diameter, may be mistaken for *B. dermatitidis*.

## IMMUNITY

Positive complement fixation tests and positive intradermal skin tests have been reported in cases of South American blastomycosis. As in North American blastomycosis, a positive complement fixation test is indicative of a spreading infection (Lacaz, 1945). It becomes negative after treatment with sulfonamides and is negative in minimal infections. Skin tests with the filtrate of Sabouraud's broth in which several strains have been grown (paracoccidioidin) as well as with heat-killed saline suspensions of the yeastlike phase of the fungus become positive in 24 to 48 hours. Of the 750 cases listed by Almeida et al. (1946), 465 occurred in the white race, 140 in the yellow race (Japanese), 43 in Mulattoes and 27 in Negroes. Such a distribution would seem to indicate that the white race is the most susceptible and the Negro the least susceptible.

## DIAGNOSIS

Pus and scrapings from the buccal mucosa and the skin lesions, pus from fluctuant nodes and smears of biopsied nodes should be examined as fresh preparations under a cover glass. Sputum in suspected pulmonary infections should also be examined. In such materials *B. brasiliensis* appears as large (10 to 60  $\mu$  in diameter), round, multiple-budding cells. The buds may be large and few in number from the surface of the parent cell and measure 10 to 30  $\mu$  in diameter or they may be small and numerous and measure 1 to 5  $\mu$  in diameter.

Materials should be cultured on blood agar at 37° C. and on Sabouraud's glucose agar at room temperature. Guinea pigs may be inoculated intratesticularly with the infected materials and cultures obtained after the development of lesions.

## TREATMENT

South American blastomycosis responds dramatically to the sulfonamides (Décourt

et al., 1946). Sulfathiazol, sulfadiazine and sulfamerazine, when given over long periods of time, have been found to be effective against the mucocutaneous, lymphatic and visceral types of infection. Penicillin was not effective against *B. brasiliensis* in vitro or in vivo (Almeida et al., 1946).

#### EPIDEMIOLOGY

South American blastomycosis is a disease of rural communities, workers in close association with farming and soil showing a high incidence of infection. Males are more frequently infected than females (10 to 1) except among the Japanese where the ratio is not so great. It is thought that the Japanese women, who work in the fields with the men, have an equal opportunity for infection. Although it is felt that the fungus must have a saprophytic existence in nature, it has not been found in the soil or on natural substrata, nor have infections of animals been reported.

#### CONTROL MEASURES

There are no known effective control measures for South American blastomycosis.

### HISTOPLASMA CAPSULATUM

#### DEFINITION

*Histoplasma capsulatum* is a small, oval, yeastlike fungus in tissues and in culture on sealed, blood agar slants at 37° C. In culture at room temperature, it is a typical moldlike filamentous fungus. It produces a disease of the reticuloendothelial system of man resembling kala-azar. This disease is known as histoplasmosis or Darling's disease.

#### HISTORY

Darling (1906) first described an intracellular organism in the tissues from natives in the Canal Zone who had died of a disease similar to visceral leishmaniasis. The organism was thought to be a protozoon

closely related to *Leishmania donovani* and was named *Histoplasma capsulatum*. Da Rocha-Lima (1913) reported budding in these forms and called the organism *Cryptococcus*. Hansmann and Schenken (1933-1934) and De Monbreun (1934) were able to culture *H. capsulatum* from their cases and proved it to be a filamentous fungus.

#### CULTIVATION

*Histoplasma capsulatum* can be cultured on all common laboratory media. On sealed blood

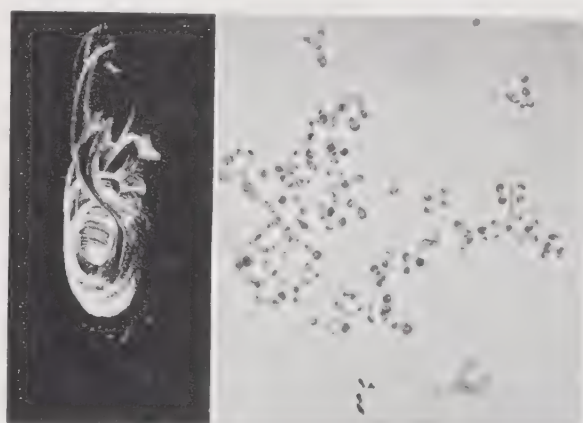


FIG. 61. *Histoplasma capsulatum*. (Left) Yeastlike growth on blood agar at 37° for five days. (Right) Small, budding, yeastlike cells from blood agar culture at 37° C.  $\times 520$ .

agar slants at 37° C., the growth is yeastlike, smooth, white to cream colored, and resembles *Staphylococcus albus* (Fig. 61 left). It is composed of small (2 to 4  $\mu$ ), oval, single budding cells (Fig. 61 right). On Sabouraud's glucose agar at room temperature, it is cottony and white at first, but becomes buff to brown with age (Fig. 62). Young cultures show branching, septate hyphae, bearing small (2.5 to 5  $\mu$ ), smooth, round to pyriform spores on short pedicles. Older cultures contain large (8 to 20  $\mu$ ), round to pyriform, thick-walled spores covered with fingerlike projections (Fig. 63). These tuberculate spores are characteristic and diagnostic for *H. capsulatum*. The filamentous culture may be converted to the yeastlike, tissue phase by subculturing to blood agar slants which, when sealed, are incubated at 37° C. Such yeastlike cultures may be maintained by subculturing to fresh blood agar slants every four to five days.



## DISTRIBUTION

Histoplasmosis has a recognized temperate and subtropical distribution with the majority of the cases occurring in the United States. What seems to be an endemic area



FIG. 62. *Histoplasma capsulatum*, 12 days on Sabouraud's glucose agar at room temperature.

in the United States would include the states of Missouri, Arkansas, Ohio, Tennessee, Indiana and Kentucky (Christie and Peterson, 1945) with scattered cases being reported from Florida, Alabama, North Carolina, Virginia, Minnesota, Michigan, Iowa and California. Outside of the United States cases have been reported from the Canal Zone, Honduras, Argentina, Brazil, South Africa and Java. Spontaneous infections have been reported in dogs (De Monbreun, 1939; Birge and Riser, 1945; Seibold, 1946) and in rodents (Olson, Bell and Emmons, 1947; Emmons et al., 1947). Mice, guinea pigs and dogs can be infected experimentally

## PATHOGENESIS

*Histoplasma capsulatum* causes an infection of the reticulo-endothelial system of man. It is not known how the organism enters the body; via the mucosa, lungs or intestinal tract. Ulcerations of the tongue, pharynx and larynx and ulcerations of the mucosa of the nose have been reported. These lesions might be mistaken for carcinoma. In the lungs, scattered miliary lesions and parenchymal changes may simulate tuberculosis. Diarrhea, hemorrhage and vomiting are symptoms referable to the intestinal tract where, at autopsy, ulceration of lymphoid tissue is found. With disseminated infection by the reticulo-endothelial cells, the typical case shows a septic

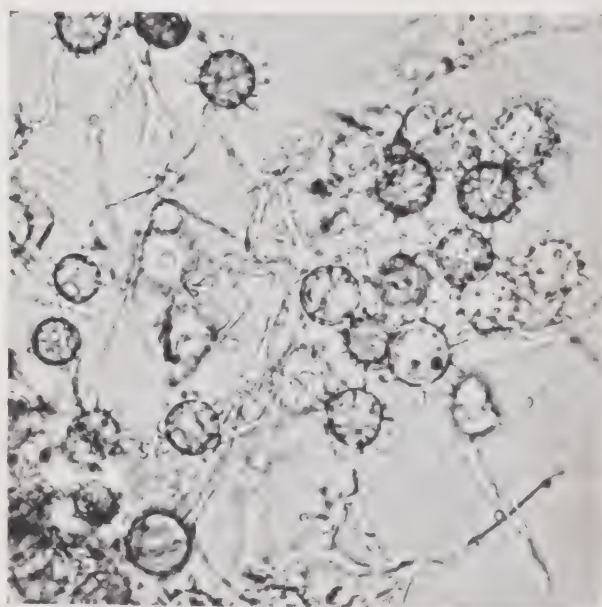


FIG. 63. *Histoplasma capsulatum*, typical, tuberculate chlamydospores developed in the Sabouraud's glucose agar cultures at room temperature. (Smith, D. T., 1947, Fungus diseases encountered in general hospital practice. American Journal of Medicine, 2, 599. Fig. 4B.)

temperature curve, splenomegaly, hepatomegaly, leukopenia, secondary anemia and emaciation. Lymphadenopathy might suggest Hodgkin's disease, lymphosarcoma or

aplastic anemia. The mortality rate is the highest of any fungus infection.

Histologically, the lesions show a central necrosis with loss of tissue and cellular structures surrounded by granulomatous tissue. The small (2 to 5  $\mu$ ) oval fungus

Extensive testing by Christie and Peterson (1945), Palmer (1945-1946) and Furcolow et al. (1946) seems to show a high correlation between pulmonary calcification in non-tuberculin reactors and positive skin tests to histoplasmin. If the skin test proves to be specific (Emmons et al., 1945; Howell, 1947) it would indicate widespread, primary, benign histoplasmosis in certain areas of the United States.

#### DIAGNOSIS

Peripheral blood smears and sternal bone marrow smears should be stained and examined for intracellular, small (2 to 5  $\mu$ ), oval bodies in the polymorphonuclear and/or mononuclear cells (Fig. 64). Lymph nodes, skin and mucosal lesions should be biopsied and sections studied for the intracellular parasite (Fig. 65). All such materials should be cultured on blood agar and Sabouraud's glucose agar at 37° C. and room temperature, respectively. Contaminated materials

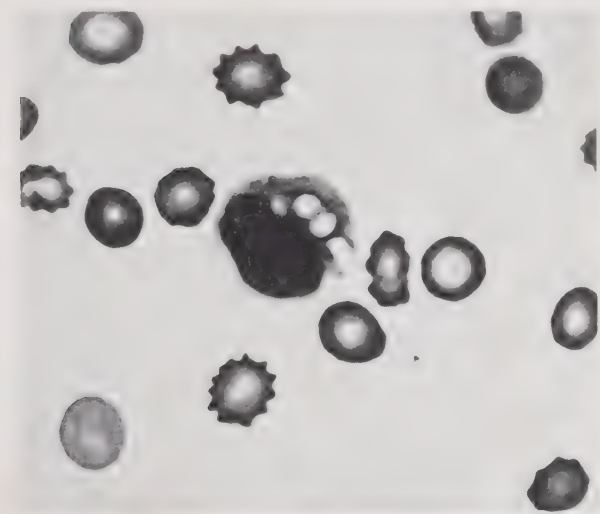


FIG. 64. *Histoplasma capsulatum*, peripheral blood smear showing *Histoplasma* in mononuclear cell.  $\times 1155$ .

appears in various phagocytic cells: large mononuclear or polymorphonuclear cells of the blood and bone marrow, endothelial wandering cells of tissues and in fixed reticulo-endothelial cells of the liver and spleen.

#### IMMUNITY

Van Pernis, Benson and Hollinger (1941) were the first to show that a patient and experimentally infected mice gave a positive skin test to filtrates of a broth in which *H. capsulatum* had been grown. A delayed tuberculinlike reaction became positive in 24 to 48 hours. Christie and Peterson (1945) reported a positive reaction in the parents of a five-months-old infected baby. Thousands of skin tests have been done with histoplasmin on people living in areas where there is known to be a high rate of nontuberculous pulmonary calcification as determined by negative tuberculin tests.

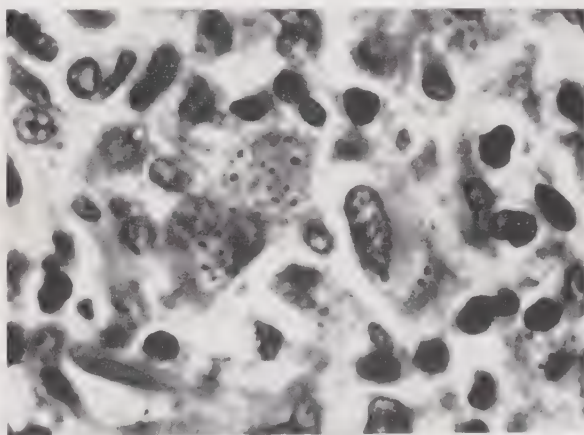


FIG. 65. *Histoplasma capsulatum*, section of gum showing intracellular bodies.  $\times 1012$ .

such as sputum, swabs from mucosal lesions and feces may be cultured on blood agar with the addition of 2 units of penicillin and 10 units of streptomycin per milliliter of medium (Thompson, 1945). Cultures may develop slowly and it may be necessary to



hold them for at least one month before colonies appear.

#### TREATMENT

Penicillin and streptomycin are of no therapeutic value in histoplasmosis. A variety of drugs have been used without success (Parsons and Zarafonitis, 1945).

#### EPIDEMIOLOGY

It is not known whether *H. capsulatum* has a natural habitat outside of the body. Infection in dogs and rodents may play some part in the transmission of the disease, but no known cases of animal to animal or animal to man transmission have been reported. Should nontuberculous calcifications be proven to be the result of a primary benign infection to *Histoplasma*, the disease might be suspected to simulate coccidioidomycosis. In histoplasmosis, however, the endemic area would include Missouri, Arkansas, Ohio, Tennessee, Indiana, and Kentucky.

There are no known measures for the control of histoplasmosis.

### SPOROTRICHUM SCHENCKII

#### DEFINITION

*Sporotrichum Schenckii* is a single-celled, fusiform, Gram-positive organism found in giant cells or polymorphonuclear cells in lesions or exudates from man and animals; cultures appear as a leathery fungus growth with characteristic spore formation. Only one species, *S. Schenckii*, is the etiologic agent of sporotrichosis, a subacute, chronic, granulomatous infection of the skin, lymph nodes and subcutaneous tissues.

#### HISTORY

Schenck (1898) first cultured and described this fungus from a patient in the United States showing refractory subcutaneous abscesses. Hektoen and Perkins

(1900), in describing the second case, named the fungus *Sporothrix Schenckii*. The disease was described by de Beurmann and Ramond (1903) in France and the fungus named *Sporotrichum Beurmanni* by Matruchot and Ramond (1905). This species and several others described from time to time are now thought to be variants of Schenck's fungus and are usually considered synonyms of *S. Schenckii*.

#### CULTIVATION

*Sporotrichum Schenckii* can be grown at room temperature or at 37° C. on all common



FIG. 66. *Sporotrichum Schenckii*, 8 days on Sabouraud's glucose agar at room temperature.

laboratory media. On Sabouraud's glucose agar at room temperature, the colonies appear as small, white growths lacking aerial mycelium (Fig. 66). As growth increases, the surface of the colony becomes folded and leathery; the color may vary from white to tan, or brown to black, depending upon the medium and the individual strain. Microscopically, the colony is composed of delicate, branching, septate hyphae, 1.5 to 2  $\mu$  in diameter. Pyriform conidia, 2 to 4 by 2 to 6  $\mu$  in size, are borne at the ends of lateral branches (conidiophores) in characteristic clusters (Fig. 67). In some

strains these conidia are also borne directly from the hyphae. On cystine agar at 37° C., the growth remains soft and yeastlike and is composed of fusiform bodies (tissue phase) and short mycelial fragments (Fig. 68).

The biochemical activities of *Sporotrichum Schenckii* vary with individual strains. Gela-

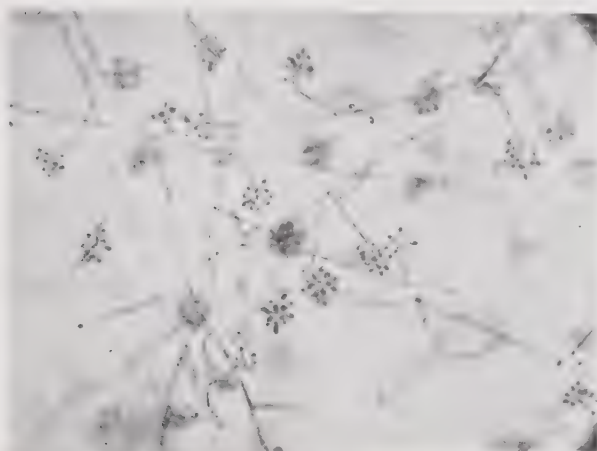


FIG. 67. *Sporotrichum Schenckii*, delicate hyphae and conidiophores with terminal clusters of pyriform conidia from Sabouraud's glucose agar.  $\times 490$ .

tine may be liquefied but only after many days; sugars, if fermented, show acid only. Milk may or may not be coagulated.

#### DISTRIBUTION

Sporotrichosis is world-wide in distribution. In the United States it is thought that endemic areas include Nebraska, Wisconsin, Kansas, the Dakotas and Missouri. Although the larger number of cases have been reported from the Mississippi Valley, it is probable that numerous cases have been recognized elsewhere in the country but have not been reported.

The fungus is widely distributed in nature and may be recovered from plants (Foerster, 1924-1926), from animals (Meyer, 1915; Anderson and Spector, 1932) and from contaminated objects such as timbers in mines (Toit, 1942). Mice and rats can be experimentally infected.

#### PATHOGENESIS

Sporotrichosis is a subacute or chronic, granulomatous infection which follows in-

troduction of the fungus by trauma. The initial lesion usually appears on exposed areas, particularly the extremities, and develops as a single lesion (pustule, ulcer, abscess or chancre) which fails to heal under ordinary treatment. With invasion of the regional lymphatics, the characteristic picture of sporotrichosis develops: ascending chronic lymphangitis with cordlike thickening of the lymph vessels and multiple subcutaneous abscesses along the course of the infected lymphatics. These abscesses are gummalike and may or may not rupture spontaneously. The epitrochlear and axillary nodes are usually not enlarged and systemic reactions are rare. The ulcerating, nonhealing, primary chancrelike lesion with lymphatic involvement suggests tularemia and/or syphilis. This is the localized lymphangitic type of infection which is most prevalent in the United States. Other clin-

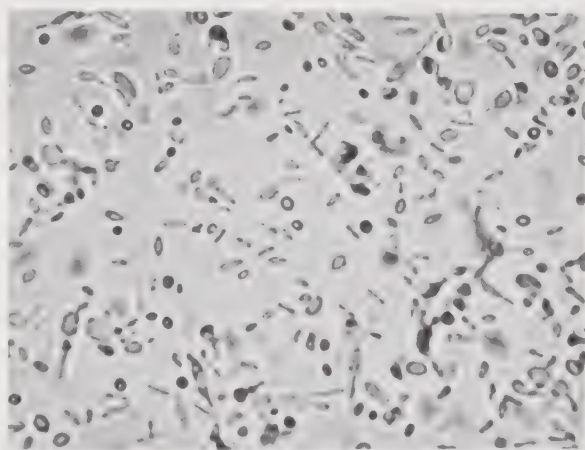


FIG. 68. *Sporotrichum Schenckii*, fusiform cells from cystine agar culture at 37° C.  $\times 580$ . (Smith, D. T., 1947, Fungus diseases encountered in general hospital practice. American Journal of Medicine, 2, 602, Fig. 7B.)

ical manifestations have been described by Beurmann and Gougerot (1912), varying from a localized lymphangitis to a widely disseminated subcutaneous or systemic gummatous sporotrichosis.

Histologically the lesions may show only a nonspecific, chronic, inflammatory process



or become granulomatous with lymphocytic infiltration, plasma cells, giant cells and fibrosis. The fusiform bodies are rarely seen in such sections or in the pus from the lesions. Therefore, diagnosis by direct examination of these materials is doubtful or impossible.

#### IMMUNITY

Agglutinins can be demonstrated in high titer, 1:600 to 1:1,000, in the serum of patients infected with *S. Schenckii* when a filtered spore suspension of the culture is used as the antigen. However, the specificity of this reaction has been questioned (du Toit, 1942), as many normal sera also give positive tests. Complement fixing antibodies can be demonstrated but the test is cumbersome. A high degree of sensitivity, beginning after about five days, can be demonstrated by using heat-killed saline suspensions of the fungus as the skin test material. A delayed tuberculinlike reaction manifests itself in 24 to 48 hours.

#### DIAGNOSIS

Positive cultures are the best means of diagnosis. The fusiform bodies that are found in experimentally infected animals are found only rarely in materials from human lesions. Pus from unruptured nodules, swabs, scrapings and biopsies of ulcerated lesions should be obtained and cultured at both room and incubator temperatures. The characteristic colony with its typical spore formation allows identification of *S. Schenckii*.

#### TREATMENT

Sporotrichosis responds readily to iodides administered orally over long periods. Recurrence can be avoided by extending the treatment one or two months after apparent cure. An occasional resistant case may respond to sulfonamides (Navarro-Martin, 1940; Noojin and Callaway, 1944).

#### EPIDEMIOLOGY

Sporotrichosis has been said (Foerster, 1926) to be an occupational disease of horticulturists, for many infections could be traced to injuries by barberry thorns. That the infection could assume a major role as an industrial disease was pointed out by du Toit (1942) in his report of 650 cases among miners and native workers in the gold mines of the Witwatersrand in South Africa. The fungus was isolated from the mine timbers and from the dust and infection usually followed injury by machines, or rock, and scratches by wire. Individuals in contact with infected rats, dogs, horses and mules may become infected. Meyer (1915) also found *Sporotrichum* on the hair and skin of normal horses, associated with infected horses. These animals as well as rats, cats and rabbits may act as passive carriers of the organism. Flies may also play an occasional part in the transmission of the fungus.

#### COCCIDIoidES IMMITIS

##### DEFINITION

*Coccidioides immitis* is a spherical, thick-walled, endospore-filled organism in tissue or exudates; and a fluffy, white, cottony fungus in culture at room temperature. It produces a highly infectious disease (known as coccidioidomycosis) with an acute, benign, primary, self-limited respiratory infection; and a chronic, malignant, secondary, progressive, disseminated infection. The secondary progressive disease is usually referred to as coccidioidal granuloma.

##### HISTORY

Posadas (1892) and Wernicke (1892) first described from Argentina what is known now to be *Coccidioides* in the tissue of a patient with lesions similar to mycosis fungoides. Rixford (1894) first described from California a protozoanlike organism in the tissue of a patient with lesions resembling tuberculosis cutis. This case and a second,

occurring in California, were reported by Rixford and Gilchrist (1896). Both patients had primary skin lesions with subsequent lymphatic dissemination resulting in death.

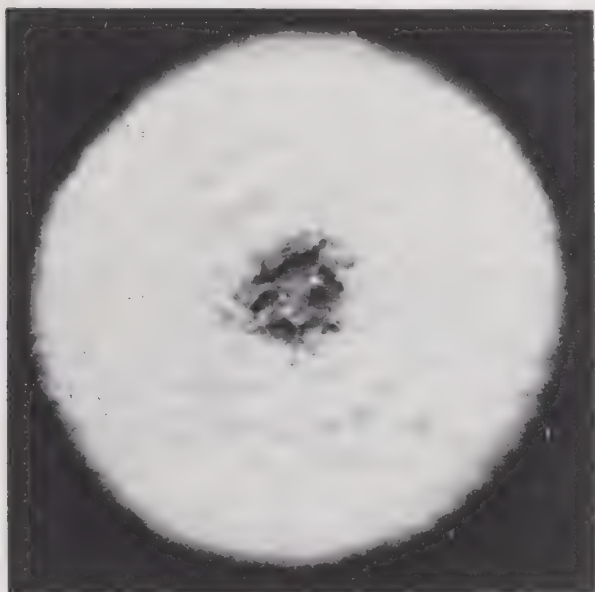


FIG. 69. *Coccidioides immitis*, 12 days on Sabouraud's glucose agar at room temperature.

At this time, the organism was named *Coccidioides immitis* and the disease became known as coccidioidal granuloma. Ophüls and Moffitt (1900), however, cultured the organism, proving it to be a filamentous fungus. Only coccidioidal granuloma was known until the benign, primary type of infection was described by Gifford (1936) and Dickson (1937a, 1937b).

#### CULTIVATION

*Coccidioides immitis* can be grown at room temperature on all common laboratory media. On Sabouraud's glucose agar at room temperature, the colony develops quickly with abundant aerial mycelium which is cottony and white at first, but may become powdery, and buff to brown with age (Fig. 69). It shows numerous arthrospores which appear as deeply staining, rectangular structures,  $2 \times 4$  to  $5 \mu$ , separated by clear spaces along the course of the hyphae (Fig. 70). In old cultures, the hyphae fragment, freeing the arthrospores, and the culture becomes friable and powdery. Cul-

tures should not be grown in Petri dishes as the powdery aerial growth, consisting of numerous arthrospores, is highly infectious. The tissue phase (spherule formation) has rarely been observed in artificial culture (Baker and Mrak, 1941). *Coccidioides immitis* liquefies gelatin in 3 days; milk is peptonized and coagulated.

#### DISTRIBUTION

Endemic regions in the United States are responsible for almost all of the infections caused by *C. immitis*. The San Joaquin Valley in California, the area around Phoenix and Tucson in Arizona, and central west Texas, constitute the highly endemic areas. Outside of the United States, occasional cases have been reported from Italy, South-eastern Europe, and the Hawaiian Islands. The Chaco region of Argentina may prove to be an endemic area: five cases have been reported (Jorge, Niño and Latienda, 1946), and it is felt that many more may exist. A detailed distribution of *C. immitis* is given by Baker, Mrak and Smith (1943). *C. immitis* also causes disease in cattle,

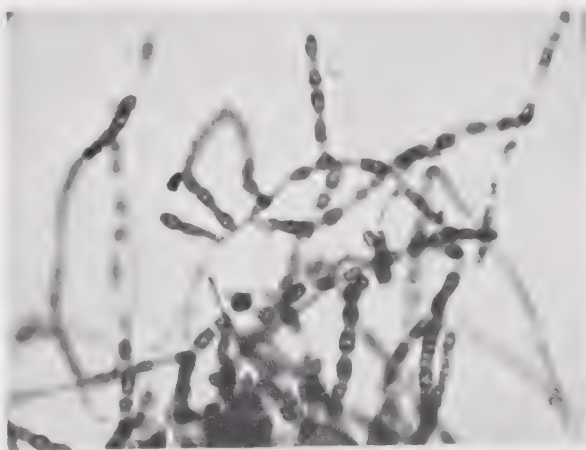


FIG. 70. *Coccidioides immitis*, hypha developing characteristic arthrospore formation from Sabouraud's glucose agar at room temperature.  $\times 490$

sheep and dogs (Stiles and Davis, 1942) and rodents (Emmons, 1942) living in the endemic areas. Most laboratory animals can be experimentally infected



## PATHOGENESIS

*C. immitis* causes an acute, benign respiratory disease which is usually self-limited in the white skinned race but has a tendency to develop into a progressive, malignant, disseminated, highly fatal disease in the dark skinned races. Primary infection usually takes place in the respiratory tract (occasionally skin abrasions) by breathing in dust containing infectious material. The infection may be subclinical or, after an incubation period of 8 to 14 days, the symptoms may be those of bronchial pneumonia or "flu": chills, fever, malaise, anorexia, cough, pleurisy, headache, backache and night sweats. About 2 to 5 per cent of such cases develop hypersensitivity which becomes evident after 5 to 14 days as typical erythema nodosum or erythema multiforme with skin lesions of from one to four weeks' duration. This form of the infection is not fatal and is known as San Joaquin Fever, Valley Fever, and Desert Rheumatism when associated with allergic manifestations.

Endogenous reinfection or dissemination at the time of the primary infection gives symptoms similar to those of tuberculosis. Lesions may appear anywhere in the body: lungs, larynx, lymph nodes, bones, joints, central nervous system, etc. This form of the infection is highly fatal and is known as coccidioidal granuloma. Histologically, the lesions are typical granulomata with tuberclelike formation indistinguishable from those seen in tuberculosis.

## IMMUNITY

Complement fixing antibodies and precipitins can be demonstrated in the serum of patients infected with *C. immitis*. The complement fixing antibodies have the same significance as in blastomycosis, i.e., indicate spreading infection and poor prognosis. Hypersensitivity to the fungus can be demonstrated by the coccidioidin skin

test. A positive test usually develops in 3 to 21 days following infection and gives a delayed tuberculinlike reaction in 24 to 48 hours.

## DIAGNOSIS

Pus and sputum should be examined as untreated, fresh preparations under a cover glass. Pleural fluid and gastric contents should be centrifuged and the sediment examined similarly. Sputum and gastric



FIG. 71. *Coccidioides immitis*, large, thick-walled endospore-filled spherule and smaller immature spherules in pus.  $\times 490$ .

contents also may be treated with cupric sulfate (0.05 per cent final concentration), allowed to stand for four hours and then centrifuged; sediment should be examined microscopically. In all materials, *C. immitis* appears as a thick-walled spherule, 10 to 80  $\mu$  in diameter, filled with endospores (Fig. 71). Infected materials should be cultured on blood agar at 37° C. and on Sabouraud's glucose agar at room temperature. A differential medium used by Smith is said to give excellent results for culturing *C. immitis* from infected materials (1 per cent ammonium chloride, 1 per cent sodium acetate, 0.8 per cent tribasic potassium phosphate, 0.04 per cent cupric sulfate and 2 per cent agar). Also, mice should be inoculated intraperitoneally and guinea pigs intratesticularly with infected materials or

suspicious cultures. Examine tissues or exudates from such animals for the characteristic spherules (Fig. 72).

#### TREATMENT

In primary coccidioidomycosis the prognosis is excellent. Treatment should be symptomatic with enforced bed rest until the temperature, sedimentation rate and white count are normal.

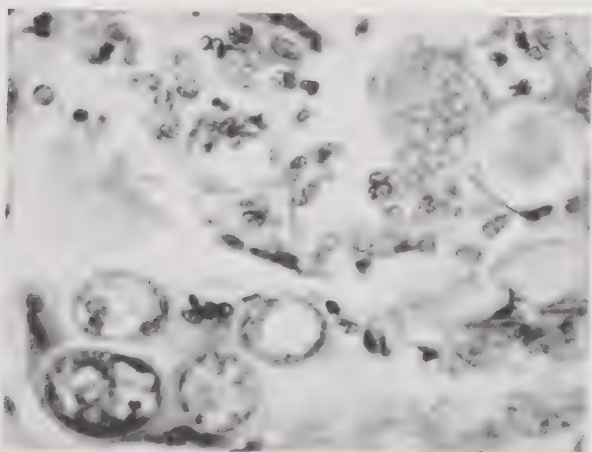


FIG. 72. *Coccidioides immitis*, section of lung showing mature and immature spherules.  $\times 490$ .

There is no effective treatment for the secondary, progressive coccidioidal granuloma. Symptomatic treatment with bed rest, high vitamin, high caloric diet may be helpful, but prognosis is poor.

#### EPIDEMIOLOGY

Coccidioidomycosis is a dust-borne disease of the arid regions of the western and southwestern parts of the United States. Inhabitants in these areas show a high incidence of infection as demonstrated by a positive coccidioidin test (Aronson et al., 1942). The infection rate of susceptibles (newcomers) follows closely a seasonal variation, most cases arising in the dry summer and autumn months when rainfall is lowest and dust more prevalent (Smith et al., 1946). *C. immitis* has been cultured from

the soil (Stewart and Meyer, 1932; Smith and Baker, 1941; Emmons, 1942). Emmons (1942) suggested that the disease may be primarily one of rodents and accidentally transferred to man by means of contaminated soil. It is thought not to be transferred from man to man or from animal to man (Smith, 1940).

#### CONTROL MEASURES

Studies carried out in conjunction with the Army Air Forces in four air fields in the San Joaquin Valley, California, during the period from 1941 to 1946 showed that dust control of such areas reduced infection rates from one-half to two-thirds in non-immune susceptibles. Paving roads and runways, planting lawns and using refined oil on athletic areas proved effective control measures (Smith et al., 1946).

### MONOSPORIUM APIOSPERMUM

#### DEFINITION

*Monosporium apiospermum* is a fungus which appears as a large, macroscopic grain or granule, white to light yellow in color, made up of wide, septate hyphae in tissue or exudates from draining sinuses. In culture, it is a gray to light buff filamentous fungus which reproduces characteristically by single spores from the ends of conidiophores. This fungus and many others (several genera and species) produce the disease known as maduromycosis or Madura foot.

#### HISTORY

The discovery of maduromycosis or mycetoma in India and the early history of this type of fungus infection has been adequately summarized by Gammel (1927). The confusion relative to the variety of etiologic agents known to cause the infection was clarified by Chalmers and Archibald (1916) when they proposed that the disease mycetoma should be classified into two categories according to the type of



fungus causing the infection: (1) Actinomycotic mycetoma caused by species of *Actinomyces* and (2) Maduromycosis caused by the higher filamentous fungi. This classi-

*Allescheria Boydii* (Shear, 1922). Emmons (1944) has shown *A. Boydii* to be the perfect stage of *Monosporium apiospermum* which has been isolated from numerous cases of maduromycosis.

#### CULTIVATION

*Monosporium apiospermum* can be cultured on all common laboratory media. On Sabouraud's glucose agar at room temperature, the colony develops quickly as a white, cottony, aerial growth which becomes grayish to buff in color (Fig. 73). A black pigment is usually produced in the agar on the reverse side of the colony.

Microscopically, single, ovoid to clavate conidia, 5 to 7 x 8 to 10  $\mu$  in size, with truncate bases, are seen on the ends of conidiophores of various lengths (Fig. 74). Coremia (bundles of hyphae) may also be seen with conidia terminating the individual hyphae coming from the bundle. In some strains, conidia are produced laterally from the hyphae and occasionally in small clusters. The usual method of spore production, however, is the development of single conidia from the ends



FIG. 73. *Monosporium apiospermum*, 15 days on Sabouraud's glucose agar at room temperature.

fication has been adopted by most investigators. *M. apiospermum* is a filamentous fungus recognized as one of the higher fungi that occasionally cause maduromycosis or Madura foot.

The first case of maduromycosis on the American continent was reported by Adami and Kirkpatrick (1895) in Montreal. No cultures were obtained from the yellowish-gray granules recovered from the lesions of their patient. The first case in the United States was reported by Hyde et al. (1896) in a patient from Iowa, but they also failed to obtain cultures from the whitish granules found in the lesions. Wright (1898), in Boston, reported the second case and was able to culture a filamentous fungus from black granules obtained from tissue. This fungus, however, was not identified. Boyd and Crutchfield (1921) cultured a filamentous fungus from one of their cases. In culture, this fungus had an ascomycetous stage as well as a conidial stage and was named

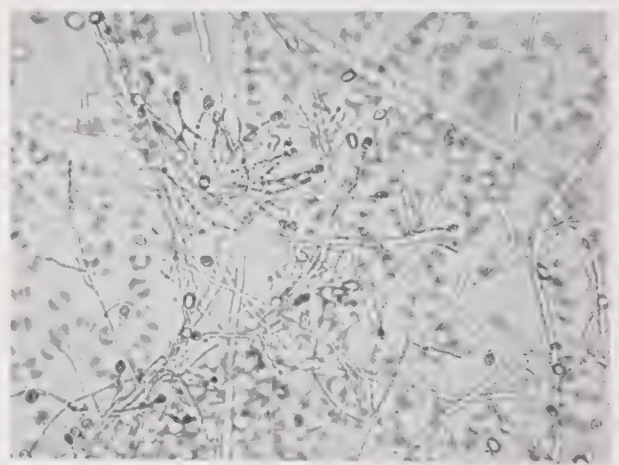


FIG. 74. *Monosporium apiospermum*, conidiophores terminated by a single conidium.  $\times 230$ . (Conant, N. F., Martin, D. S., Smith, D. T., Baker, R. D., and Callaway, J. L., 1944, Manual of Clinical Mycology. Philadelphia, Saunders, p. 187.)

of conidiophores. The ascomycetous phase of *Monosporium apiospermum* has been described for only two strains: *Allescheria Boydii* isolated by Boyd and Crutchfield (1921) and *Monosporium apiospermum* iso-

lated by Dowding (1935). In these cultures, the cleistothecia (closed perithecia) 50 to 200  $\mu$  in diameter, are thin-walled, brownish structures containing subglobose asci in each of which are seen 8 elliptical, faintly brown-walled ascospores, 4 to 4.5 x 7 to 7.5  $\mu$  in size.

When two differently named fungi are found to be the conidial and ascosporic phase of the same fungus, taxonomists usually discard the name given to the conidial phase and retain the name given to the ascosporic phase. Since all the isolates of *Monosporium apiospermum*, with the exception of the two isolates mentioned, have produced only conidia by which they might be identified, this familiar name has been retained for convenience. All isolates which produce perithecia and ascospores, however, should be identified as *Allescheria Boydii*.

#### DISTRIBUTION

Maduromycosis is endemic in India and the majority of the cases reported elsewhere in the world have been found in the tropics. Summaries of the distribution of this type of fungus infection and lists of the multiplicity of fungi which have been isolated are contained in reports by Boyd and Crutchfield (1921), Gammel (1927), Brindley and Howell (1932) and Conant et al. (1944).

In the United States, *Monosporium apiospermum* has been reported from cases of maduromycosis in Texas, Massachusetts, Georgia (2), Maryland, North Carolina and Pennsylvania. Outside of the United States, *M. apiospermum* has been reported from cases of maduromycosis in Canada, Virgin Islands, Paraguay, Argentina, Brazil (2), Algeria and Italy. So far as is known, *M. apiospermum* causes only human infections. No spontaneous infections in animals have been reported, and there have been no reports of successful experimental infections in laboratory animals.

#### PATHOGENESIS

Maduromycosis is a chronic, slowly progressive, unilateral infection of the subcutaneous tissues caused by the introduction

of one of several different filamentous fungi by trauma. The majority of cases have occurred on the foot, but occasional infections of the leg and hand have been described (Shaw and Macgregor, 1935; Symmers and Sporer, 1944). Infection usually follows an injury which heals and, after varying periods of time, becomes noticeable by the formation of papules, deep-seated nodules or abscesses which rupture to form multiple draining sinuses. In some instances the infection begins with swelling and pain and the subsequent development of indurated areas which become open fistulae from which drains serosanguineous fluid containing the characteristic granules. With extension of the infection to the fascia, muscles and bone, and the development of dense fibrous tissue, the foot becomes swollen, club-shaped and markedly deformed. Osteomyelitis of the bones of the foot may cause extensive fusion of these structures resulting in stiffness and loss of motion. There is usually no systemic reaction to the infection and little, if any, pain.

Histologically, the lesions are similar to those of actinomycosis. Abscess formation is prominent. Granules situated in the pus may be surrounded by a granulation tissue composed of polymorphonuclear cells, plasma cells, lymphocytes, eosinophils and macrophages. Giant cells may or may not be present. The granules are rounded to lobulated structures composed of wide, septate hyphae with chlamydospores around the periphery.

In the case reported by Symmers and Sporer (1944) the granules were small, composed of clusters of pigmented chlamydospores surrounded by granulomas with numerous giant cells showing phagocytosis of fungus particles. So far as is known no immune bodies have been demonstrated in maduromycosis.

#### DIAGNOSIS

Materials from fistulae and draining sinuses and biopsy specimens should be exam-



ined for granules. These are oval, lobulated, 0.5 to 2 mm. in diameter, white to light yellow granules when *M. apiospermum* is the infecting fungus. Other fungi may produce black, red, or orchid colored granules. Microscopically, the maduromycotic type of granule is composed of wide, branching, septate hyphae, 2 to 4  $\mu$  in diameter, with numerous chlamydospores (Fig. 75).

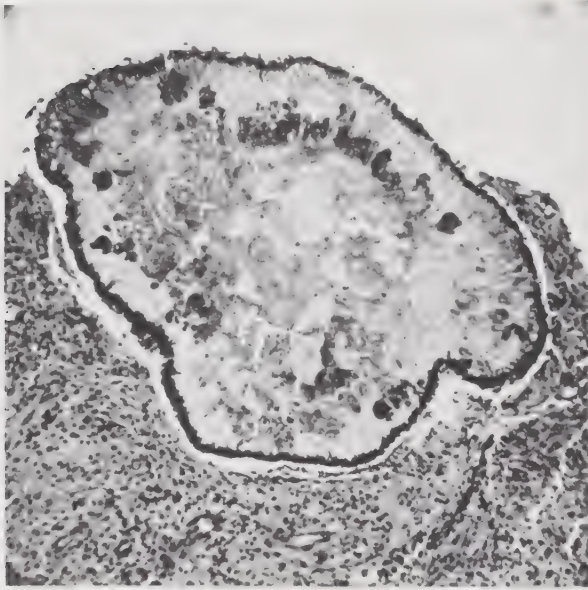


FIG. 75. *Monosporium apiospermum*, section from subcutaneous tissue showing granule. (Smith, D. T., 1947, Fungus diseases encountered in general hospital practice. American Journal of Medicine, 2, 602, Fig. 8.)

The actinomycotic type of granule, on the other hand, is composed of fine, delicate, nonseptate hyphae, 1  $\mu$  or less in diameter. These two types of granules should be distinguished in microscopic preparations for a correct diagnosis.

The granules should be washed in several changes of sterile saline or broth before culturing to avoid contamination. Aspirated material should be cultured directly. Granules should be cultured on Sabouraud's glucose agar or beef infusion glucose agar at room temperature and the cultures should be kept for at least two weeks.

#### TREATMENT

Sulfonamides and penicillin have been tried without success. In one case caused by a *Cephalosporium*, of two years' duration, in which the infection was confined to the skin and subcutaneous tissue, Twining et al. (1946) reported apparent cure with penicillin. Chemotherapy, however, can prevent or cure secondary bacterial infection of the multiple draining sinuses. The infected member usually has to be amputated.

#### EPIDEMIOLOGY

Maduromycosis is a disease of the tropics and subtropics with occasional cases reported in the temperate zone. It is a disease of the exposed parts of the body, particularly of the feet. Most of the fungi belong to genera known to contain saprophytic species which may be cultured from the soil. Injuries to barefoot laborers and others going without shoes predisposes to infection. Men are more commonly infected than women; all races are susceptible.

#### CONTROL MEASURES

From the nature of the infection, the type of fungi involved and the almost inevitable history of injury to bare feet, it would seem that wearing shoes would be a practical control measure.

#### HORMODENDRUM PEDROSOI

##### DEFINITION

*Hormodendrum Pedrosoi* is a small, round, thick-walled, dark brown body found in crusts, exudates and tissue where it reproduces by splitting. In culture it is a dark green to brown or black filamentous fungus which reproduces by a variety of spore forms. This fungus with at least two others (*Hormodendrum compactum* and *Phialophora verrucosa*) produce the disease known as chromoblastomycosis.

## HISTORY

Pedroso (1911) in Brazil observed dark bodies in the tissue from a patient with verrucous skin lesions but failed to identify the fungus which he isolated. Later, Pedroso and Gomes (1920) reported four Brazilian cases, including Pedroso's original patient, and named the etiologic agent *Phialophora verrucosa*. This identification was based on an earlier report by Lane (1915) and Medlar (1915) in which they described *P. verrucosa*, a fungus isolated from nodular lesions on the buttocks of an Italian in Boston in whose tissue they also had observed dark-brown splitting bodies. Brumpt (1922) restudied the South American fungus and named it *Hormodendrum Pedrosoi* and Terra et al. (1922) called the disease chromoblastomycosis. At this time, two fungi, *Phialophora verrucosa* in the United States and *Hormodendrum Pedrosoi* in South America, were considered to be the etiologic agents of the disease. Moore and Almeida (1935), however, when re-examining the South American cultures, found one of the original isolations of Pedroso to be *P. verrucosa*. Martin, Baker and Conant (1936) reported the first case caused by *H. Pedrosoi* in the United States. Carrion (1935) described *Hormodendrum compactum* from Puerto Rico as the third fungus to cause chromoblastomycosis.

While *P. verrucosa* and *H. compactum* have remained constant in their morphology, *H. Pedrosoi* has varied considerably and has presented a real problem in classification. Since *H. Pedrosoi* has been isolated from the majority of the cases of chromoblastomycosis, this has led to numerous attempts to reclassify the fungus (Moore and Almeida, 1936; Carrion, 1942; Binford et al., 1944).

## CULTIVATION

*Hormodendrum Pedrosoi* can be cultured on all common laboratory media. On Sabouraud's glucose agar, the colonies are dark green to

brown or black, covered with a feltlike aerial mycelium, with individual strains showing variation in rate of growth and gross character of the colony (Fig. 76 left). Microscopically, *H. Pedrosoi* varies greatly in its

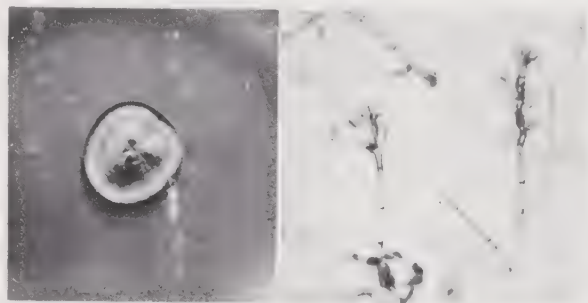


FIG. 76. *Hormodendrum Pedrosoi*. (Left) Twenty-one days on Sabouraud's glucose agar at room temperature; (center) *Hormodendrum*-type of conidiophore,  $\times 440$ ; (right) *Acrotheca*-type of conidiophore.  $\times 440$ . (Right and center, Conant, N. F., Martin, D. S., Smith, D. T., Baker, R. D., and Callaway, J. L., 1944, Manual of Clinical Mycology. Philadelphia, Saunders, p. 100.)

method of conidial formation. Three different methods of sporulation are recognized: conidia in branching chain formation from conidiophores of varying length (*Hormodendrum* type) (Fig. 76 center), conidia surrounding

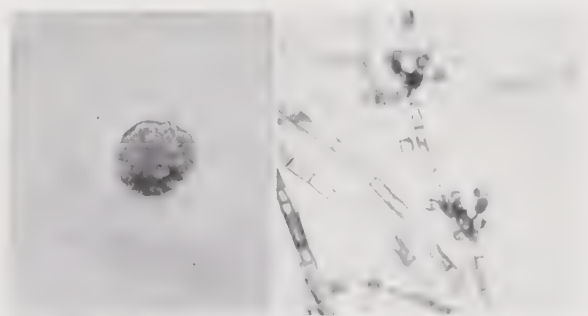


FIG. 77. *Hormodendrum compactum*. (Left) Thirty-eight days on Sabouraud's glucose agar at room temperature. (Right) Conidiophore with compact spore head.  $\times 470$ .

the swollen, knotted, club-shaped, terminal ends of hyphae (*Acrotheca* type) (Fig. 76 right), and conidia produced semiendogenously from flask-shaped conidiophores with a terminal cup (*Phialophora* type). Different



strains show a predominance of one type of sporulation.

*Hormodendrum compactum* grows slowly on Sabouraud's glucose agar producing a heaped, brittle colony, olive black in color with tufts of coarse aerial mycelium (Fig. 77 left). Microscopically, chains of spherical to subspherical conidia in compact sporulating heads distinguishes this species from *H. Pedrosoi* (Fig. 77 right). The *Phialophora* type of sporulation is also found in cultures of *H. compactum*.



FIG. 78. *Phialophora verrucosa*. (Left) Twenty-eight days on Sabouraud's glucose agar at room temperature; (right) typical conidiophore.  $\times 410$ .

*Phialophora verrucosa*, on Sabouraud's glucose agar, produces a dark brown to black colony with short, feltlike, olivaceous, gray aerial mycelium (Fig. 78 left). This fungus reproduces by a single type of sporulation. Microscopically, flask-shaped conidiophores, borne terminally or laterally from the hyphae, produce conidia singly at the tip within a cup-like structure (Fig. 78 right). These conidia, successively produced at the tip of the conidiophore, usually remain in clusters in undisturbed preparations.

#### DISTRIBUTION

Chromoblastomycosis is world-wide in distribution, with the exception of Asia. Although the disease is found in temperate climates, the majority of cases have occurred in the tropics. A review by Weidman and Rosenthal (1941) plus more recent data show the following distribution: United States [Boston, Fort Worth, Texas, St. Louis (3), Durham, N. C. (2), Philadelphia, Atlanta, and Miami, Florida (2)]; West Indies [Cuba (31), Puerto Rico (9) and the Dominican Republic]; Central America [Costa Rica (3), Guatemala and

Panama (3)]; South America [Brazil (60+), Venezuela, Argentina, Paraguay and Uruguay]; Europe [Russia (3)]; Africa [Rhodesia, Algeria and the Union of South Africa (12)]; East Indies [Java and Sumatra (2)]; Japan (3). Of these cases only one has been caused by *H. compactum* (Carrion, 1935, Puerto Rico), six have been caused by *P. verrucosa* [Lane, 1915 (Boston); Pedroso and Gomes, 1920 (Brazil); Wilson et al., 1933 (Texas); MacKinnon, 1934 (Uruguay); Moore and Mapother, 1940 (St. Louis); Moore et al., 1943 (St. Louis)] and the rest have been caused by *H. Pedrosoi*.

So far as is known, chromoblastomycosis is a human disease; there have been no reports of spontaneous infections in animals. Monkeys, dogs, rabbits, guinea pigs, rats and mice have been inoculated by various routes with only occasional reports of experimental infection (Mello, Leao and Cury, 1946).

#### PATHOGENESIS

Chromoblastomycosis is a chronic, slowly developing (months to years), unilateral, granulomatous infection of the skin which develops following introduction of the fungus by trauma. In the majority of cases the lesions appear on the lower extremities, but occasional cases have been reported where lesions appeared on the hand, arm, face, neck and buttock (Weidman and Rosenthal, 1941). In the typical case, the lesions are unilateral and are first noted as warty growths which extend slowly as satellite lesions along the lymphatics. Over a period of months the lesions enlarge to become vegetating, papillomatous, verrucous, elevated nodules with a cauliflowerlike appearance. Elephantiasis of the affected member usually results from marked fibrosis and lymph stasis. Metastases have been reported in only two cases (Carrion, 1938; Montpellier and Catanei, 1927). Systemic infections are unknown and regional adenopathy results only from secondary bacterial infection. The protean nature of the infection is apparent (Pardo-Castello et al., 1942) when

it is seen that lesions lacking the typical appearance described above must be differentiated from other mycotic diseases, tuberculosis verrucosa cutis, late nodular syphiloderm, epithelioma, leishmaniasis, yaws, mossy foot, etc.

Histologically the lesions are granulomatous with pseudotubercle formation. The epidermis shows marked hyperplasia, extensive hyperkeratosis, parakeratosis and acanthosis with elongated, distorted rete pegs. The corium contains pseudotubercles made up of epithelioid cells and giant cells of the Langhan's type in the center of which are polymorphonuclear leukocytes. Dark-brown fungus bodies may be found among the polymorphonuclear cells or in the giant cells. Surrounding these tuberculoid granuloma are lymphocytes, plasma cells, eosinophiles and a few polymorphonuclear cells.

#### IMMUNITY

Positive complement fixation tests have been reported by Balina et al. (1932) and Conant and Martin (1937). Montpellier and Catanei (1927), however, reported a negative complement-fixation test and a negative agglutination test with the serum of their patient. Since both these tests are difficult to perform, they are not practical diagnostic aids. Diagnosis is more readily established by finding the organism.

#### DIAGNOSIS

Epidermal debris from the cauliflowerlike nodules should be examined in ten per cent potassium hydroxide preparations for the presence of the dark-brown, septate bodies, 6 to 12  $\mu$  in diameter, characteristic for chromoblastomycosis. Sections from biopsies also should be examined for the presence of these bodies and the typical histopathologic reaction (Fig. 79). Both these materials should be cultured on Sabouraud's glucose agar at room temperature. Since the three recognized etiologic agents (*H. Pe-*

*drosoi*, *H. compactum* and *P. verrucosa*) produce identical bodies in tissue, the causative agent of a given case can be established only by the isolation and identification of the fungus.

#### TREATMENT

Small, localized lesions may be removed successfully by surgical excision, cauterization or electrocoagulation. Iodides by mouth

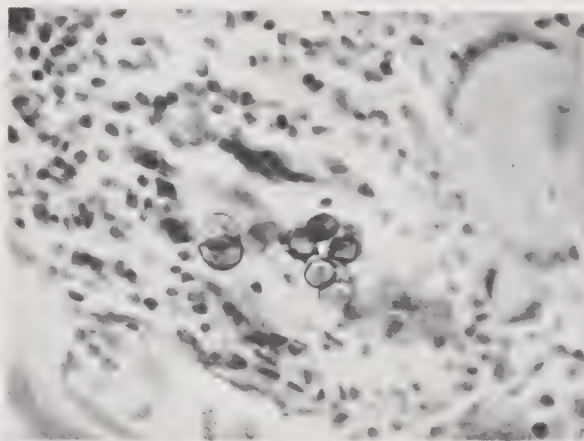


FIG. 79. *Hormodendrum Pedrosoi*, section of skin showing pigmented, round, splitting bodies of fungus in giant cell.  $\times 495$ .

or intravenously with or without X-ray treatment locally have been used with variable results. One extensive case was treated by iontophoresis with copper sulfate with good results (Martin, Baker and Conant, 1936).

#### EPIDEMIOLOGY

Chromoblastomycosis is a disease of the skin of the exposed parts of the body. It is most frequent in the tropics among barefooted, agricultural laborers and others with close contact with the soil. The fungi are saprophytes in nature (Conant, 1937) and enter the skin by trauma. The disease is not transmitted from man to man, is more prevalent during adult life (30 to 50 years of age), is rarely reported in females and shows no racial immunity.

There are no known control measures.



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## 33

# The Bacteriology of Mucous Membranes

### INTRODUCTION

The mucous membranes harbor, regularly and transiently, a varied group of micro-organisms, including pathogens whose presence is associated with disease or with a carrier state, indigenous parasites and saprophytes introduced from the outside world. The third group need not concern us, while the first and many members of the second, having been dealt with elsewhere in this book, need not be treated here at length. The present chapter will deal chiefly with the characters and pathogenic behavior of certain indigenous parasites which are peculiar to the "normal flora" of the body.

### THE BACTERIA OF MUCOUS MEMBRANES

#### RELATIVES OF PATHOGENS

Micro-organisms having little or no pathogenicity, but, belonging to groups that include frank pathogens, are conspicuous members of the flora of all mucous membranes. Included under this head are the genera *Staphylococcus* and *Micrococcus*, particularly in the nose; *Streptococcus*, on all mucous membranes, but especially those of mouth and throat; *Neisseria*, *Corynebacterium* and *Hemophilus* in the nasopharyngeal area; *Treponema* and *Borrelia*, most characteristic of the mouth; *Mycobacterium*, on the external genitalia; and *Escherichia*, *Aerobacter*, *Proteus*, *Pseudo-*

*monas* and *Clostridium* in the large intestine.

#### THE DISTINCTIVE INDIGENOUS BACTERIA

The following groups of micro-organisms are rarely or never found in the environment, but are peculiar to the normal flora of the body.

The anaerobic streptococci consist of a somewhat heterogeneous group of Gram-positive cocci growing in chains and including strictly anaerobic members and others that develop a tolerance for oxygen after artificial cultivation. They occur on the mucous membranes of the alimentary, respiratory and genito-urinary tracts. Although pure cultures of them are usually nonpathogenic for laboratory animals, they may play a rôle in the pathology of mixed infections. Anaerobic streptococci have been incriminated in a type of endogenous puerperal septicemia that may follow difficult or traumatic labor (Colebrook, 1930, 1931).

The genus *Veillonella* (family *Neisseriaceae*) consists of strictly anaerobic small Gram-negative cocci, occurring in irregular clusters and apparently nonsaccharolytic. They have been recovered from the mouth and pharynx, from liver abscess, and from putrid gangrene in several areas, but their pathogenic significance, if any, is uncertain. One of them, *V. alcalescens* has been implicated in subacute bacterial endocarditis.

The anaerobic *Vibrios* consist of anaerobic actively motile comma-shaped or spiral bacteria, apparently including both Gram-positive and Gram-negative members which occur in the mouth, in the large intestine, and on the mucous and mucocutaneous surfaces of the external genitalia. No systematic study of this group has been reported. Pathogenicity has been attributed to them, particularly in combination with other anaerobes (page 633).

Parasitic members of the genus *Lactobacillus* including both aerobic (*L. acidophilus*) and anaerobic (*L. bifidus*) forms, are found in the mouth and vagina, and less prominently in the intestinal tract, except in nurselings, in whom *L. bifidus* is the predominant fecal organism. They are Gram-positive, non-spore-bearing, nonmotile rods characterized by morphologic variability and instability in cultures, obligately saccharolytic, acidogenic and "aciduric," i.e., tolerating a low pH (in the neighborhood of 5.0) in the medium. A close relationship seems to exist between *L. acidophilus* and *L. casei* isolated from milk and milk products. Indirect evidence implicates the lactobacilli as inciting causes of dental caries. On the other hand, their occurrence in the vagina has been associated with freedom from infection, as will be noted below.

The genus *Bacteroides*, which seems related to and may merge with *Fusobacterium*, consists of Gram-negative pleomorphic rods and filaments, strictly anaerobic, nonsporulating, nonmotile. They are the predominant organisms in adult feces. Among recognized species are *Bact. vulgatus*, presumably nonpathogenic; *Bact. melaninogenicum*, producing black colonies on hemoglobin-containing media; and three species for which well-developed pathogenicity has been reported: *Bact. fragilis*, *Bact. funduliformis* and *Bact. necrophorus*. The last two are believed to be closely related if not identical, although the specific name *necrophorus* has been applied principally to strains exhibiting more active pathogenicity.

The bacteroides have been recovered from putrid gangrenous diseases along with other anaerobes of mucous membranes. They have also been found in the blood in a group of "septicopyemias" following peritonsillar abscess and other mucous membrane lesions. Clinically, these diseases closely resemble the anaerobic streptococcus septicemias (Smith and Ropes, 1941).

The fusiform bacilli are strictly anaerobic, nonmotile, nonsporulating Gram-negative organisms especially characteristic of the gingival area of the mouth, where they occur both in health and, in much greater profusion, in association with low-grade inflammatory processes. With them are found several varieties of spirochetes and numerous other bacteria. A similar group of organisms occurs in tonsillar crypts, in the large intestine and on the external genitalia. Fusiform bacilli are typically slender, with either clear or distinctly granular cytoplasm, and with tapering, pointed ends; but in cultures they may show much variation in morphology, including forms that resemble bacteroides. Although several species have been described, Bøe (1941) was unable to find consistent groupings among 30 strains. *Fusobacterium*, in addition to the characteristics mentioned, produces indol and  $H_2S$  and is only weakly saccharolytic (final pH above 6.0). It is generally nonpathogenic in pure culture, but appears to be a contributing pathogen in mixed infections.

*L. buccalis*, the only clearly defined species in the genus *Leptotrichia*, is a characteristic parasite of the mouth. It is an unbranched, coarse Gram-positive filamentous organism which tends to fragment into rods that may simulate fusiform bacilli, since the bacillary forms are rapidly decolorized by alcohol in all but very young cultures. The leptotrichia can be distinguished from fusobacteria and also from lactobacilli by serologic reactions, by virtue of their failure to produce indol or  $H_2S$ , and by their intermediate acidogenic capacity (final pH 4.7



to 5.2). They seem to be less strictly anaerobic than fusiform bacilli and are not known to be pathogenic.

*Dialister pneumosintes*, isolated from nasopharyngeal washings of very early cases of influenza, is listed by the S.A.B. Committee as an anaerobic member of the Tribe *Hemophilae*, but seems closely similar to the bacteroides except for its filterability through Berkefeld V and N candles. Olitsky and Gates (1922), who first described it, also described several other Gram-negative anaerobic bacteria, which, except for initial filterability, on isolation seem to correspond with the anaerobic vibrios, veillonella and bacteroides. Mills, Shibley and Dochez (1928), who reported similar findings, found that filterability, lost after several artificial cultures, could be restored by passage through chick embryos.

Many other micro-organisms whose identity remains obscure have been described in cultures or in direct microscopic preparations from mucous membranes; and there is no reason to believe that the foregoing listing is complete. Gram-positive anaerobic bacilli, described as bacteroides or under new generic names, have not been clearly distinguished from the lactobacilli or from *Actinomyces* (Chapter 31). The taxonomic position of such large motile organisms as "*Spirillum sputigenum*" and "*Fusocillus girans*" remains inadequately characterized. Along with certain bizarre micro-organisms like a "test-tube-brush" form ("*Vibriothrix tonsillaris*"), they can be found in fresh preparations and dried films, but they have seldom been cultivated and much remains to be learned about them.

#### DISTRIBUTION OF MICRO-ORGANISMS ON THE MUCOUS MEMBRANES

The data on relative prominence of indigenous micro-organisms on representative mucous membranes given in Table 51 are only approximate, since quantitative infor-

mation on this subject is very incomplete. Among the forms listed, the micrococci (including *Gaffkya* and *Sarcina*) and the aerobic spore-bearing rods (*Bacillus*) may owe their presence in these areas to their ubiquity in the environment. The listing of staphylococci, beta streptococci, pneumococci and *Hemophilus*, as well as of the viruses, unavoidably fails to distinguish between indigenous forms and pathogens. The group-D hemolytic streptococci of the intestine, and perhaps the virus of herpes, seem to belong in the former category. The other groups, including yeasts and protozoa, are intended to refer to the less pathogenic or nonpathogenic members of each genus or group. The "L" organisms are those found associated with bacteroides, or isolated from the genital tract, by Dienes (1940, 1942) and others.

The most varied flora is found in the mouth, especially in the normally narrow and shallow clefts between gums and teeth in which the anaerobic forms flourish. Both the pharyngeal and the intestinal flora are fed from the mouth, but in each of these areas local conditions evidently favor different types, e.g., the Gram-negative diplococci in the pharynx, and the coliforms and bacteroides in the intestine. The flora of the vulva and the preputial area is probably derived in large part from intestinal contamination, and has certain resemblances to both the intestinal and the oral floras. The eye, nose and vagina harbor fewer varieties than the preceding areas. The nasolachrymal duct, the accessory nasal sinuses and the respiratory tract below the pharynx, as well as the internal genito-urinary tract, all contain very few bacteria and seem to have no characteristic normal flora. The same may be said of the healthy empty stomach, while the flora of the small intestine, which seems to consist principally of aerobic bacteria, becomes progressively richer and more varied toward the large intestine, where it reaches full development.

TABLE 51. DISTRIBUTION OF MICRO-ORGANISMS ON THE MUCOUS MEMBRANES

GENUS OR GROUP	EYE	NOSE	PHARYNX	MOUTH	INTESTINE	EXTERNAL GENITALIA	VAGINA
Micrococcus		*	*	*	*	*	*
STAPHYLOCOCCUS	*	+	*	*	*	*	*
ALPHA, GAMMA STREPTOCOCCUS		*	++	++	+	+	+
Beta Streptococcus		*	*	*	+ <sup>1</sup>		
ANAEROBIC STREPTOCOCCUS....			*	+	*	*	*
Pneumococcus		*	*	*			
NEISSERIA		*	++	*			
VEILLONELLA			*	+		*	
Bacillus			*	*	*		
CLOSTRIDIUM					+		
CORYNEBACTERIUM.....	*	+	*	*		+	+
LACTOBACILLUS.....				+	+		++ <sup>2</sup>
ACTINOMYCES			+	+	*		
Leptotrichia				+			
Mycobacterium				*	++	+	*
ESCHERICHIA					*		
Aerobacter					*		
Klebsiella					*		
Proteus				*	*	*	
Pseudomonas					*	*	
Hemophilus		*	*	*			
Dialister.....			*	*	++	*	
BACTEROIDES.....			*	+	*	+	
FUSOBACTERIUM			*	+	*	*	
ANAEROBIC VIBRIO			*	++	+	+	
SPIROCHETES				+	*	*	+
Yeasts.....				*	*	*	*
Protozoa				*		*	
"L" organisms.....				*		*	
Viruses		*	*	*		*	

The more important indigenous forms are given in capitals.  
\* Irregular.  
+ Common.

++ Prominent or constant.  
<sup>1</sup> Group D hemolytic enterococci.  
<sup>2</sup> During the period of ovarian activity.

SIGNIFICANCE OF THE BACTERIA OF MUCOUS MEMBRANES

SOURCES AND MODES OF DEVELOPMENT

All the mucous membranes are sterile at birth. Since many of the indigenous bacterial forms are not found in nature apart from the mucous membranes, it is probable that their derivation in one individual is principally from the mucous membranes of others. The mouth appears to be the first area to become contaminated after birth (in 6 to 10 hours), and, as suggested above, may become an important primary source of micro-organisms for the other areas.

Although it may be contaminated directly from the maternal vagina and vulva, the development of the complete flora waits for the eruption of the teeth and must have more varied sources.

The flora is almost exclusively aerobic at first, with greening streptococci apparently predominant. Lactobacilli have been recovered toward the end of the second week but thereafter tend to disappear until the teeth have erupted. The full flora, and particularly its characteristic anaerobic members, seem to require the gingival crevice, since it is greatly reduced in both the preeruptive and the edentulous mouth.



In the intestine bacteria appear toward the end of the first day after birth. In the breast-fed infant there is an early "phase of developing infection" (Tissier, 1900) during which white staphylococci, colon bacilli, spore-bearing anaerobes, enterococci and other bacteria appear in increasing numbers and then disappear rapidly. In this "phase of transformation" the anaerobic *Lactobacillus bifidus* comes by the third or fourth day to constitute as much as 99 per cent of the total organisms of the feces. In bottle-fed infants the phase of developing infection is more prolonged, reaching its peak toward the fourth day; there is no well-marked transformation, and *L. bifidus*, although present, is never prominent.

A somewhat similar transformation takes place in the vagina. *L. acidophilus* makes its appearance at about the third day after birth, apparently favored by the strong acidity of the sterile neonatal vaginal secretion, and persists for a few weeks in association with a simple flora. Thereafter, as the secretion becomes scanty and more alkaline, the flora becomes more varied and predominantly coccal. At puberty there is a sudden reversion to a simple "aciduric" flora (largely lactobacilli and yeasts), associated with the deposition of glycogen in the vaginal wall apparently as the result of ovarian activity. The low pH of the secretion resulting from the fermentation of glycogen by lactobacilli is looked upon as a defense mechanism which prevents establishment in the vagina of foreign and possibly harmful bacteria (Cruickshank and Sharman, 1934). After the menopause the prepubertal scanty alkaline secretion and varied flora return.

#### ACTIVITIES FAVORABLE TO THE HOST

Success in the rearing of animals as diverse as the chick and the monkey under aseptic conditions (Reyniers, 1943) leaves no doubt that "normal" parasites are not essential to the life of the host. In "germ-free"

animals, however, almost any chance contaminant (e.g., *B. subtilis*) may serve as an active pathogen; hence, it appears that under ordinary conditions the presence of the surface flora is associated with the development of resistance to it and may in consequence be regarded as beneficial to the host. Beyond this, the hypothesis is widely held that the presence of the indigenous flora on any given mucous membrane may be a factor in preventing the establishment of nonindigenous forms; but very little is known about the mechanism of such effects. We have seen that lactobacilli in the vagina during the period of ovarian activity, and perhaps in the intestinal tract of nurselings, may retard the growth of other microorganisms. There is evidence, moreover, that "inhibin" (Dold and Weigmann, 1934)—a salivary factor inhibitory to the growth of the diphtheria bacillus—may be produced by salivary streptococci of the "mitis" type (Muhlenbach, 1939). It remains to be determined whether other specific instances of bacterial antagonism are to be found on the mucous membranes.

Another type of favorable activity of the indigenous flora is the synthesis of vitamins and other foods by intestinal bacteria. Among the substances known to be thus synthesized are thiamine, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, biotin, folic acid, vitamins E and K, and perhaps amino acids or even whole proteins, although the nutritional value to the host of the bacterial substance is not established. Data obtained in deficient feeding experiments (Greaves, 1939; Najjar et al., 1943, 1944), and also in the course of administration of sulfaguanidine or succinyl-sulfathiazole, leave little doubt that some at least of the vitamins thus formed are utilized in human nutrition, and that certain toxic effects of sulfonamides may be due to interference with the synthesizing activities of the intestinal bacteria. On the other hand, it must be pointed out that ascorbic acid is decomposed by *E. coli* and *A. aerogenes*

(Young et al., 1942, 1943), a fact which may explain why oral vitamin C therapy has sometimes failed whereas subsequent injection proved effective.

#### PATHOGENIC EFFECTS OF THE INDIGENOUS FLORA

Although the presence of indigenous parasites is not incompatible with continued good health and may even contribute to it, these agents can also play a significant part in disease processes. For example, *Clostridium welchii* and *Escherichia coli* are evidently harmless while in the lumen of the intact intestine, but serious disease may result from implantation of the former into necrotic tissue, or of the latter into a normally sterile mucous or serous area such as the urinary tract or the peritoneal cavity.

Predisposing conditions in the host are required for the development of infection due to the micro-organisms of mucous membranes. Thus, the alpha streptococci of the mouth and other areas are enabled to produce subacute endocarditis when persons with pre-existing cardiac defects are exposed to a transient bacteremia resulting from surgical or other manipulations, for example, during the extraction of teeth (Rosebury, 1944). Actinomycosis (Chapter 31) appears to be an analogous endogenous infection; and the pathogenesis of both the puerperal septicemia associated with anaerobic streptococci and the postanginal "septicopyemia" from which *Bacteroides* species have been recovered, in so far as it is understood, follows a comparable pattern. In the two latter instances a suspicion of mixed infections has not been resolved by the use of possibly selective cultural methods, and the diseases in question may merge with a large and as yet poorly defined category in which much evidence points to a complex of nonsporulating anaerobes as the infective agency (Smith, 1932). In this group are Vincent's stomatitis and other diseases of

the supporting tissues of the teeth; similar processes in the pharyngeal and genital areas; lung abscess, pulmonary gangrene, certain forms of appendicitis, intestinal ulceration or perforation, liver abscess and other putrid necrotizing processes of the respiratory and intestinal tracts; and, among other entities, a common form of tropical ulcer of the skin. These are the so-called fusospirochetal diseases. Their common characteristics are necrosis and a foul fibrinous exudation containing spirochetes resembling those of the mouth, fusiform bacilli, streptococci including strict anaerobes, and anaerobic vibrios. Many additional micro-organisms may be present, their variety and prominence varying with the location of the lesions and with other conditions. Unpurified exudates from such disease processes, when inoculated into the tissues of guinea pigs or other animals, produce transmissible lesions which show the common characteristics mentioned. Repeated passage tends to eliminate all but the four varieties of micro-organisms named. Smith (1930) has shown that mixtures of the following four pure cultures were capable of producing the same pathologic process: *Treponema microdentium*, a fusiform bacillus, an anaerobic streptococcus and anaerobic vibrio. On the other hand, these and other species tested individually or in incomplete combinations yielded a variety of pathologic effects but failed to reproduce the characteristic picture of fusospirochetal infection.

The findings suggest a concept of *focal infection* markedly different from views of Rosenow and others who associated a wide range of generally chronic, symptomless local infections assumed to be caused by anhemolytic streptococci in or adjacent to the mucous membranes (e.g., at the root-ends of teeth) with a similarly broad category of diseases of obscure etiology (e.g., rheumatoid arthritis). This view gave rise during the 1920's and 1930's to an enthusiastic wave of tooth extractions, tonsillec-



tomies and comparable operations intended for the cure or prevention of the presumed "focal" infections. Although the generally disappointing or even deleterious results of such operations led to the discarding of this concept, it can hardly be doubted that diseases of more or less remote tissues can, in fact, be caused by certain bacteria of the mucous membranes. The emerging view, however, would look upon these as separate entities, each of which is, like subacute endocarditis, actinomycosis or the fusospirochetal infections, distinctive in its bacteriology and doubtless also in its pathogenesis.

#### PREDISPOSITION AND NATURAL RESISTANCE

There is much evidence that the intact and healthy mucous membranes not only repel nonindigenous micro-organisms, but also limit the proliferation of and prevent invasion by the normal flora. While the defensive mechanisms concerned with this function are not fully understood, it seems clear that they include the production of such substances as lysozyme and other bacteria-inhibiting and virus-inhibiting agencies, including the gastric acidity (Chapter 32), as well as the action, noted previously, of certain bacteria in limiting the development of others. They also embrace a complex group of factors which determine the integrity of tissues and cells. Tissue or cellular damage or impairment is the prime prerequisite for endogenous infection, whether such damage acts merely to contaminate a normally sterile area or, as seems more commonly true, provides conditions that permit normally harmless bacteria to proliferate unduly and become invasive. The agencies inducing such damage or impairment may be listed and briefly exemplified as follows:

**Trauma.** The fact that simple wounds of the mouth almost always heal without infection suggests that simple trauma of a mucous membrane is unimportant in itself.

Trauma in difficult labor, however, is regarded as responsible for endogenous puerperal septicemia associated with anaerobic streptococci, and a stab wound perforating the large intestine may result in peritonitis. Contamination of a skin wound with the intestinal or oral flora (the latter in human bite wounds) may result in severe infection. Mild but long continued irritation has clearly recognizable infective sequels in the mouth, as is shown by the prompt recovery from simple gingivitis that usually follows removal of accumulated food residues or tartar from the teeth. Such irritation is evidently a complicating factor in more serious oral disease, but of itself it appears to induce only mild and localized effects.

**Intoxication.** Disturbances of the gingival area of the mouth commonly associated with intensive antisiphilitic therapy with mercury or bismuth seem either to predispose to endogenous infection or to aggravate a pre-existing infection. The hyperplastic gingivitis that may follow dilantin therapy for epilepsy suggests a comparable infective component. That the mucous membrane infection occurring in agranulocytosis due to sulfonamides, arsphenamines or thiouracil may be a serious complication of this disease is indicated by recoveries following treatment with penicillin (Boland et al., 1946).

**Nutritional Deficiency.** Infection complicates several deficiency states, notably with vitamins A, C and several members of the B group. In many, although by no means all, instances the infection is evidently endogenous, but the bacteriology of such processes has not been adequately studied. The xerophthalmia of vitamin-A deficiency seems to be an endogenous infection resulting from interference with secretion of lysozyme (Findlay, 1925). Symptoms referable to fusospirochetal infection are found in the mouth and probably occur in other mucous membranes in severe scurvy and pellagra, and experimentally in folic

acid deficiency. It is commonly believed that vitamin deficiencies contribute to many low-grade mucous membrane infections, presumably of endogenous origin, but such a nutritional mechanism appears to be neither specific nor invariable. Noma, a rapidly spreading fusospirochetal gangrene of mouth or vulva, is clearly associated with malnutrition. It is of interest that this highly fatal disease has been found, like agranulocytosis, to respond to penicillin therapy, here supplemental to restored nutrition.

**Infection.** That acute infectious disease may predispose to secondary endogenous infection is illustrated by the view of Ariel (1934) that intestinal perforation in typhoid fever or in intestinal tuberculosis is due to the resident fusosprochetal flora, and, by the repeated observation that genital fusospirochetosis is often secondary to chancroid, lymphogranuloma venereum or granu-

loma inguinale. If the virus of herpes may be looked upon as indigenous, recurrent herpes simplex accompanying febrile infections would be another example of this phenomenon.

**Other Predisposing Agencies.** Sensitization by repeated auto-inoculation is thought to be a factor in the pathogenesis of actinomycosis (Chapter 31). Certain endocrine disturbances, notably diabetes (Rudy and Cohen, 1939) and those associated with pregnancy (Ziskin and Nesse, 1946) induce changes in the mucous membrane in which endogenous infection is a complicating phenomenon. The etiology of the two most common diseases of the mouth, dental caries and "pyorrhea alveolaris," is thought to involve endogenous infection made possible by a complex of predisposing factors (Rosebury, 1944b, 1947).

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## 34

# Principles of Sterilization

### GENERAL

#### DEFINITIONS

*Asepsis* refers to a technic which avoids the introduction of viable micro-organisms. *Sterilization* and *disinfection* are processes which eliminate viable microbes; the terms are essentially synonymous, but the latter is usually limited to the use of chemicals which render infectious organisms non-viable. *Antibacterial* effects are divided into *bacteriostasis*, or reversible inhibition of the multiplication of bacteria, and irreversible *bactericidal* action, which "kills" them. The terms *disinfectant*, *germicide*, and *bactericide* are synonyms for bactericidal agents. *Antiseptics* are antibacterial substances which can be applied to body surfaces, cavities, or wounds to prevent or combat bacterial infection; these compounds do not necessarily completely sterilize the treated surface. *Chemotherapeutics* are antibacterial (or, more generally, antimicrobial) substances which are sufficiently nontoxic to the host to permit their use in the treatment of infections within the tissues as well as on body surfaces.

#### CRITERIA OF DEATH

The fundamental criterion of the death of a microbe is its inability to propagate when placed in a favorable environment.

The word "sterile," usually applied only to materials freed of viable bacteria, therefore, also describes precisely the state of the non-viable bacterial cells, and will be used in this chapter in preference to "dead" or "killed." Changes in morphology, staining properties and motility are only irregularly correlated with loss of viability, while respiration and other measurable enzymic activities are often quite unchanged for some time following sterilization by means, such as ultraviolet light, which do not grossly alter the protoplasm. Autolysis following sterilization by certain agents occurs with only a few bacterial species, such as pneumococcus, and hence is a sufficient but not a necessary criterion of death. It may also be pointed out that *sterilization* is not identical with the *destruction* of bacteria or their products, though the terms are often loosely interchanged. It is necessary, for example, to take pains that solutions prepared for intravenous administration be not only sterile but also free from the pyrogenic products of previous bacterial contamination.

A rigorous distinction between a bactericidal and a bacteriostatic effect is difficult to establish in practice. For one thing, in the presence of water at ordinary temperatures all living beings require active metabolism in order to maintain their organization in the face of the universal tend-



ency toward disorder, such as protein denaturation; in consequence, when the harmoniously integrated metabolism of bacteria is inhibited by a bacteriostatic agent, the cells gradually die, just as they do in an exhausted or inadequate medium. It is convenient to define a true bactericidal effect as a more rapid process than this. Furthermore, since the death of bacteria is recognized only by their failure to propagate when inoculated into a suitable medium, the decision as to whether bacteria are sterile will sometimes vary strikingly with the medium employed. For example, as early as 1889 Geppert observed that Koch had overestimated the disinfectant action of  $\text{HgCl}_2$ , since apparently sterile anthrax spores could be revived after exposure for a week or more if they were washed with a solution of  $\text{H}_2\text{S}$ , which forms an insoluble salt with the mercuric ion and hence reverses its equilibrium with components of the bacterial protoplasm. Recently Fildes has shown that the process of reversal can be carried still further by cultivation in media containing sulfhydryl compounds, such as cysteine or thioglycollic acid. Even in animals, administration of an appropriate sulfhydryl compound (BAL) has been found to permit infection by mercury-treated pathogenic organisms which were harmless to untreated animals. A similar but less striking effect has been reported by McIlwain *in vitro* for bacteria apparently killed by brief exposure to acridine dyes, and then "resurrected" by subsequent exposure to nucleic acids.

It is perhaps better not to attempt to define sterility or death in absolute terms, but rather to define it in operational terms, subject to change with the development of new technics. A material is sterile if it is impossible by available technics to demonstrate viable bacteria in it. As a practical matter, pathogenic bacteria have been adequately sterilized if they are unable to

propagate in the animal body. It is theoretically possible that reversal of the apparent bactericidal effect of disinfectants other than those mentioned may simply await the discovery of the proper reagents. On the other hand, even in the cases described where such reagents are available, enough exposure to the disinfectant leads eventually to irreversible change. It would be a mistake to abandon the concepts of bacteriostatic and bactericidal action, simply because their definition is relative. The distinction is important in understanding the mode of action of chemotherapeutics.

### HISTORY

Practical sterilization arose from empirical observations on the preservation of food by drying or salting, and the Egyptian success in embalming with essential oils. Koch, in 1871, first studied disinfection by  $\text{HgCl}_2$  with pure cultures, and Krönig and Paul in a classic paper in 1897 produced quantitative data which showed the gradual nature of the process. The field remains, however, largely empirical, and while it is of great practical and commercial importance, recent developments in research have been limited. Most of the interest in antibacterial agents has naturally been occupied with chemotherapy.

The quantitative data possible in studies in disinfection, however, lend themselves to theoretical interpretation. The study of the "pharmacology" of bacterial cells has therefore not been altogether neglected. Two recent scientific developments lead us to expect a revival of interest in the essentially physicochemical problem of the mechanism of disinfection. One is the increasing possibility of studying, at the level of structural chemistry, interactions of proteins with small molecules. These are postulated to underly most pharmacologic reactions, and disinfection in particular. The other development is the widespread interest in the study of cytochemistry

and biochemical genetics, in which disinfectant as well as chemotherapeutic agents will undoubtedly be very important reagents.

#### DIFFERENTIAL SUSCEPTIBILITY

The various bacterial groups differ widely in their sensitivity to heat and to various disinfectants but not to radiation; they range from the pneumococcus, which tends to autolyze in all but the most favorable environments, to the tubercle bacillus, which resists strong acid and alkali. The reactions may be quite specific, however; the tubercle bacillus is much more susceptible than most bacteria to fatty acids (soaps). Within a given genus the species behave quite similarly. While generalizations are frequently made concerning the behavior of the classes of bacteria, particularly the difference in the susceptibility of Gram-positive and Gram-negative bacteria to various disinfectants, extensive testing generally reveals striking exceptions. The differences in susceptibility are only relative, the more resistant organisms requiring a more intense or prolonged exposure to the sterilizing agent. It must also be remembered that the process of sterilization is gradual. The exposure of sputum to an appropriate concentration of alkali, for example, may sterilize all the cells of most bacterial species present, while the tubercle bacilli and some staphylococci and streptococci are more resistant. Yet, the vast majority of the tubercle bacilli themselves may be sterilized, the successful diagnostic cultivation depending on the viable residue.

The susceptibility of the cells of a given species varies markedly with their physiologic state. The cells in a young culture, which are larger and more rapidly growing than those in an old culture approaching nutritive exhaustion, are more susceptible to various physical and chemical agents. There may well also be a difference in the susceptibility of the cells at different ages

between birth and fission, but this cannot be measured since the cells in all cultures are of mixed ages. Much more important than these effects, from a practical point of view, is the difference between vegetative cells and the spores formed by some species; spores are much more resistant to abnormal temperatures and to chemical agents but not necessarily to radiation. They therefore seem to have the biologic function of permitting survival through hard times. The nature of the organization of spores which provides this increased resistance is poorly understood. They are said to have thicker cell walls, which could hinder the penetration of chemicals but could not be responsible for their resistance to heat. They also have a slightly lower water content, which may be concerned with protein denaturation. It is likely that the major reasons for the resistance of spores are too subtle for detection by present technics. As a practical matter, sterilization procedures must be adequate for the spores which may be present; the vegetative forms are then automatically taken care of.

The rate of sterilization of bacteria depends on a large number of variables: nature and concentration of the disinfectant, species and physiologic state of the bacteria, concentration of exposed bacteria, temperature, pH, presence and concentration of protective materials, and technic of detecting survival. The usual method of measuring disinfectant action consists of mixing a given inoculum with a given concentration of disinfectant and removing at successive intervals a loopful or other small sample which is transferred to a large volume of broth or plated on a solid medium; either the rate of decrease in the colonies yielded on this subculture, or the time of disappearance of viable bacteria, is recorded. Since data are available for only a small and arbitrary fraction of the possible permutations of these variables, the action of the



various agents will be discussed in a general way with little quantitative detail.

## PHYSICAL AGENTS

### MOIST HEAT

This is the sterilizing method of choice for all materials except those which would be damaged by the heat or moisture. It is rapid, all organisms are susceptible, and it penetrates clumps and reaches surfaces that might be missed by a chemical disinfectant. The vegetative cells of all pathogenic bacteria, as well as fungi and viruses, are sterilized within a few minutes at 50° to 70° C., and even the much more resistant spores of the anthrax bacillus and other pathogens are all sterilized within a few minutes at 100° C. In consequence, syringes, needles and instruments for minor surgery may be sterilized when necessary by boiling for from 10 to 15 minutes. Since the sterilizing action of hot water is enhanced several fold by weak (e.g., N/7,000) acid or alkali, a little washing soda increases its efficiency. The process has a remarkably high temperature coefficient, the rate of killing increasing several hundred fold with a temperature increase of 10° C.

There are, however, thermophilic saprophytes which have the remarkable property of surviving and even multiplying rapidly at temperatures as high as 80° C.; their spores can survive boiling for 24 hours. For surgery and bacteriologic cultivation, free of contamination, absolute sterility is essential. For this purpose Pasteur introduced the use of steam under pressure; the autoclave has since become the symbol of the bacteriologic laboratory. In order to insure sterilization of the most resistant spores that may be present, it has become customary to expose material to steam at a temperature of 120° C. for 20 to 30 minutes. This temperature is attained by steam at a pressure of 15 pounds (in excess of atmospheric pressure) at altitudes near sea level;

at high altitudes slightly higher pressures are necessary.

In using an autoclave, it is important that flowing steam be allowed to displace the air before building up pressure, for steam mixed with air (superheated steam) fails to penetrate porous material as rapidly as saturated steam, and it heats up objects much more slowly since the steam cannot condense on surfaces unless saturated. The condensation of steam permits even large objects to reach the ambient temperature within a few minutes. It is also important that vessels be loosely plugged or capped and not completely filled with liquid, in order to permit free ebullition of the air contained. Modern autoclaves are provided with a steam jacket which makes it possible to keep the contents of the autoclave hot after replacing the steam with air, in order to dry off the condensed water rapidly. This is essential for cloth-wrapped or cotton-plugged objects, since these materials are effective bacterial filters only when dry.

Some media contain components which would be rapidly destroyed at 120° C. but can withstand 100° C. These media are sterilized by exposure to flowing steam at atmospheric pressure in an Arnold sterilizer on 3 successive days. The theory underlying this *fractional sterilization* (Tyndallisation) is that the vegetative cells will be destroyed at 100° C. and the spores will germinate during the intervals of storage. The procedure is obviously applicable only to nutrient media which will promote germination. A modification is the inspissation at 85° C. of egg media for tubercle bacilli. Fractional sterilization was widely used for the complex older media, but is much less commonly used today.

The process of *pasteurization* which was introduced by Pasteur to prevent the acidification of wine by bacterial fermentation following its alcoholic fermentation by yeast, has found much wider use as a means of preventing milk-borne infection. It consists of heating at 62° C. for 30 minutes. Since

the phosphatase in the milk is inactivated by this amount of heating, a negative phosphatase reaction is a test of the adequacy of the exposure. The effectiveness of pasteurization depends upon the fact that none of the common milk-borne pathogens (tubercle bacilli, salmonella, streptococcus and brucella) form spores.

### DRY HEAT

In the absence of moisture bacteria, like isolated proteins, are much more resistant to the effects of heat. Indeed, vegetative typhoid bacilli, absolutely dry and sealed in a vacuum, are said to survive 30 minutes at 115° C. Ordinary air-dried organisms are somewhat more susceptible, but not as susceptible as when surrounded by water or steam. In consequence, sterilization by dry heat requires 160° C. for one to one and one-half hours. This temperature ruins most fabrics and somewhat weakens cotton plugs. Dry heat has the further disadvantage that hot air penetrates porous materials much more slowly than steam, so that the center of a large package of surgical dressings may not reach even 100° C. after an hour in an oven at 160° C. Sterilization by dry heat is ordinarily used only for glassware and metal objects. Intense dry heat is used in the process of flaming, which is indispensable in the bacteriologic laboratory, and in disposing of infectious materials by incineration.

It is often desirable to sterilize within a few minutes instruments unexpectedly required in the course of a surgical operation. This may be accomplished by high-pressure autoclaving (moist heat) or immersion in hot oil (dry heat); it is necessary to use oil with a high flash point.

### MECHANISM OF STERILIZATION BY HEAT

The fundamental action by which heat sterilizes bacteria has been definitely known since Harriet Chick showed in 1910 that it

parallels in several respects the heat denaturation and coagulation of proteins. Both these processes (1) have an exponential time-action curve (which will be discussed in detail later); (2) have a temperature coefficient of several hundred ( $Q_{10}$  = the ratio of the rate of a given temperature to the rate at a temperature 10° C. lower) in contrast to the  $Q_{10}$  of 2 to 3 observed for most chemical reactions; and (3) occur in approximately the same range of temperature. Even the anomalous resistance of thermophiles and spores has some parallel in protein chemistry, since some enzymes (e.g., myokinase, ribonuclease) are not inactivated by boiling under certain conditions. Further parallelism is shown in the much greater resistance to heat when in the dry state, and in the moderate protection afforded to both bacteria and proteins by the addition of a high concentration of a neutral substance, such as glucose, to the surrounding medium. The bacterial proteins include, of course, all the enzymes, as well as the nucleoproteins which are presumably genetically active; whether any particular types of bacterial proteins are especially susceptible is unknown.

The expression "thermal death time" has often been used to denote a characteristic of various bacterial species, but it cannot be precisely defined. The rate of sterilization of bacteria at a given temperature can be accurately measured, but there is no sharp end point for the death of the last cell. There is a family of values rather than a value for each species, since each temperature requires a different time. The earlier concept of the "thermal death point," which neglected the time parameter entirely, has been abandoned.

Efforts to develop relatively heat-resistant strains of bacteria by repeated subinoculation of the survivors of heating have failed to produce a really significant effect, in contrast to the successful development of drug resistance by analogous experiments.



## COLD

Most bacteria are resistant to cold, and in a nonnutrient menstruum will survive much longer in the refrigerator than in the incubator. The gonococcus and meningococcus, however, are said to die quite quickly in the refrigerator. Freezing kills approximately 50 per cent of a suspension of *E. coli*, but this is undoubtedly a mechanical effect of the freezing or the thawing, since repeated freezing and thawing is much more destructive than maintenance of the frozen culture. Further cooling to temperatures as low as that of liquid air ( $-186^{\circ}\text{C.}$ ) may cause no harm. A useful method of indefinitely preserving strains of bacteria, viruses and even cells as complicated as spirochetes or plasmodia is storage in sealed ampoules surrounded by  $\text{CO}_2$  ice ( $-78^{\circ}\text{C.}$ ). At temperatures of  $-5^{\circ}$  to  $-25^{\circ}\text{C.}$ , such as are provided by mechanical freezers, bacteria do not remain viable indefinitely, since these temperatures exceed the eutectic point of the salts present and therefore permit chemical changes in the tiny pockets of saturated salt solution which are present.

## DESICCATION

Especially from the frozen state (lyophilization), desiccation is a particularly useful means of preserving viable bacteria and sera, since no refrigeration is necessary for storage. Spirochetes appear to be uniformly killed by this procedure though not by freezing; dried plasma, therefore, probably cannot transmit syphilis.

## ULTRAVIOLET RADIATION

The sterilizing effect of sunlight on bacteria was early found to be due to its content of ultraviolet light. The spectrum of visible light ranges from a wave length of 4,000 Å (violet) to 7,500 Å (red), 1 Ångström unit (Å) being  $10^{-8}$  cm. or 0.1 millimicron ( $\text{m}\mu$ ). By far the greatest proportion of the

ultraviolet radiation emitted by the sun is screened out by the ozone present in the outer ranges of atmosphere; were this not so, most of the biologic species present on the earth's surface could not survive. The lower limit of wave length of sunlight reaching the earth's surface is approximately 2,900 Å. Practically all the ultraviolet light of sunlight is screened out by ordinary window glass, as is shown by the failure of such light to produce sunburn. While no sharp upper limit can be placed to the bactericidal wave length of light, appreciably rapid sterilization is first shown at 3,300 Å and increases rapidly with decrease in wave length. Concentrated ultraviolet light of predominantly shorter wave lengths, 2,400 to 2,800 Å, which are very much more bactericidal per unit of radiant energy than the ultraviolet components of sunlight, can be produced by mercury vapor lamps. In experimental exposure of bacteria to such light it is necessary to use vessels of fused quartz (silica), which does not absorb light of these wave lengths. (Cf. Hollaender, 1942.)

Sterilization by ultraviolet light is subject to the laws of photochemistry. (1) The mere passage of light through a transparent medium is without chemical effect; only absorbed radiant energy can be effective. (2) Light is absorbed in minimal units of energy, called quanta. The energy of a quantum is inversely proportional to its wave length; a molecule which has absorbed a quantum of ultraviolet light has consequently received a greater increment of energy, capable of activating a chemical reaction, than a molecule which has absorbed a quantum of visible light. (3) The number of quanta absorbed is proportional to the product of the intensity and the duration of the radiation, as well as to the absorption coefficient of the irradiated material. There is consequently no basis for the belief that bacterial vaccines killed by very brief exposure to intense radiation are less damaged as antigens than those killed by a comparable amount of radiation spread over a longer period of time.

The energy of the absorbed quantum, which activates molecules by producing increased interatomic vibration or else excitation of electrons to a higher energy level, may follow a

variety of paths. The activated molecule may rupture any of a variety of chemical bonds and form new bonds with adjacent molecules, or it may transfer most of its extra energy by collision to an adjacent molecule which then undergoes chemical reaction, or the energy may be entirely dissipated by collision as increased translational energy (heat) without any chemical change. Ultraviolet radiation causes formation of ozone ( $O_3$ ) in air, and hydrogen peroxide ( $H_2O_2$ ) in water containing dissolved oxygen. For this reason, intensely irradiated media are transiently toxic to subsequently inoculated bacteria.

While absorption of ultraviolet light by oxygen does occur, with formation of toxic peroxides, the absorption coefficient of certain bacterial constituents is so much greater that peroxide formation is probably a negligible source of sterilization in comparison with direct absorption by the irradiated bacteria. For this reason the absence of oxygen does not affect the rate of sterilization appreciably. The rate is also essentially independent of temperature.

The constituents of protoplasm chiefly responsible for ultraviolet absorption are the nucleic acids and nucleotides, in which the purine and pyrimidine rings give rise to a very high absorption peak at 2,600 Å, and the proteins, which have a fairly high absorption maximum at 2,800 Å due to the ring structures of tryptophane, tyrosine, and phenylalanine. The sterilization action spectrum parallels the absorption spectrum of the protein and nucleic acid of bacteria, indicating that absorption by either of these groups of compounds may have a lethal effect. From the mode of action of irradiation it is obvious that bacteria could not vary as markedly in their susceptibility to ultraviolet or X-ray irradiation as they do in their susceptibility to disinfectants.

The chemical effect of the absorption cannot be precisely defined; as was indicated above, a wide variety of changes take place. Studies of irradiated solutions have shown depolymerization of nucleic acids, and both disaggregation and aggregation of proteins which are included in the vague term denaturation.

The quantum yield of ultraviolet sterilization is small; the average *B. coli* cell has absorbed over  $10^6$  quanta by the time it is sterilized, and even the smallest viruses, which are killed by a single radiation-induced ionization (discussed later), require many ultraviolet quanta. This means that the vast majority of the absorbed quanta are dissipated without chemical reaction or that most of the molecules or chemical groups affected are not essential for survival; undoubtedly both factors are involved. Ultraviolet irradiation has been shown to produce mutations in microorganisms as well as in the germ cells of higher animals. The concept of the sterilizing effect as a lethal mutation, due to alteration of the ultraviolet absorbing nucleoproteins of chromosomes or of their analogues in bacteria, will be further discussed later (Lea, 1947).

Inexpensive mercury vapor lamps are now commercially available as sources of ultraviolet radiation. They have been used in the preparation of bacterial and virus vaccines, on the assumption that killing by this means causes less destruction of desirable antigens than the use of heat or chemical agents; this recent development remains to be evaluated. In preparing such vaccines it is important, in order to avoid occasional survival of pathogenic bacteria, that the suspensions be free of clumps, be stirred sufficiently to insure uniform exposure, and be subjected to enough radiation to provide a generous measure of safety.

Ultraviolet lamps have begun to find use in preventing air-borne infection in public places and surgical operating rooms; they have been shown to decrease cross infection in hospital wards and in animal colonies. In the laboratory they are useful in preventing contamination and decreasing the chance of infection of workers by the invisible droplets which are often scattered in the course of bacteriological transfer. While bacteria in glass tubes and pipettes may be briefly exposed to direct irradiation during transfer without harm, this is not permissible with open plates or loops. Since even brief exposure of the cornea causes severe irritation, with a latent period of about 12 hours,



the eyes must be protected from any direct exposure or from prolonged exposure to reflection from glass.

It has been shown that tuberculous cross infection in rabbits, caused by dust from bedding contaminated by urine, can be completely prevented by ultraviolet lamps; it is not necessary that a wall of ultraviolet light be formed between the cages, but is sufficient if the air is exposed to enough radiation in the course of circulating through the room (Lurie, 1945). Surfaces are sterilized, however, only by direct exposure. Although the practice of attaching lamps to public toilet seats appears to be based more on commercial enterprise than on hygienic principles, ultraviolet lamps do have real epidemiologic value and may find increasing use in the future. The patients who have become accustomed to the "germicidal" smell of phenol and essential oils in physicians' offices will probably be educated to enjoy the faint but more significant odor of ozone.

#### PHOTODYNAMIC SENSITIZATION (Blum, 1941)

Certain dyes, such as methylene blue, rose bengal, and eosin, sterilize bacteria and viruses in strong visible light, at concentrations of dye much lower than those required in the dark. Under similar circumstances they hemolyze red blood cells and denature proteins, including toxins and antibodies. The effect is produced by a comparatively small number of dyes, which vary in structure but have in common the property of fluorescence. Photosensitization has not been of much practical value in bacteriology, but must be borne in mind when working with potentially photosensitizing dyes.

These dyes, in contrast to nonfluorescent dyes, have the capacity to retain an absorbed quantum for a comparatively long time ( $10^{-6}$  to  $10^{-8}$  sec.) and then release a large fraction of its energy in a single unit either by fluorescent radiation or by transfer by collision with another molecule; the latter may then undergo chemical change. Since free oxygen is required for the reactions listed above (in contrast

with the effect of ultraviolet radiation), it appears that the dye activates either an oxygen molecule or a substrate molecule and thereby brings about the oxidation of otherwise stable substances.

Visible light, when very intense, is capable of killing bacteria, presumably via photosensitizing substances; riboflavine and porphyrins are known examples of physiologically occurring photosensitizers. This effect makes it inadvisable to expose bacterial cultures to direct sunlight, even when protected by glass.

#### X-RAYS AND OTHER IONIZING RADIATIONS

X-rays, and the  $\gamma$ -rays of radioactive elements, are electromagnetic radiations of wave length 0.01 to 10 Å; they are lethal to bacteria. X-rays are not of much practical use in bacteriology; experimentally they are of more interest because of their capacity to produce mutations than because of their lethal effect. They are sometimes used clinically in the treatment of chronic bacterial and fungus infections, but since the doses used are too low to effect much sterilization, any beneficial effect probably depends upon a tissue response.

Other ionizing radiations include cathode rays and radioactive  $\beta$ -rays, which are beams of high speed electrons, and radioactive  $\alpha$ -rays, which are high-speed helium nuclei. The quantum energy values of all these radiations are hundreds to thousands of times as great as those of ultraviolet light at 2,600 Å; consequently their mode of action is entirely different. Instead of having each quantum absorbed by a molecule of appropriate configuration, a part of the energy of a quantum is contributed to each of several hundred atoms of any kind which lie in its path, regardless of the chemical structures in which those atoms are involved. The absorbing atoms are ionized by the ejection of an electron, which is the reason for the collective term ionizing radiations. The molecules containing the ionized atoms then undergo chemical change involving the rupture and formation of bonds. The ultimate results are much the same as those caused by ultraviolet radiation, except that

(1) they are chemically more varied, (2) the energies involved are so high that there are no "duds," and (3) a single quantum produces numerous molecular changes. In consequence a single quantum may inactivate more than one bacterium or virus particle. Statistical calculation of the volume distribution of the ionizations resulting from a given amount of irradiation, and the proportion of virus particles sterilized, has shown that the "sensitive volume" of a unit of one of the smaller viruses is approximately the same as the known size of the virus particle. In other words, an ionization anywhere within that particle is lethal. With the larger viruses only a part of the total particle is lethally sensitive, and in the case of bacteria a very small fraction of the total volume. The ionizing radiations have been widely used to produce genetic mutations. The biologic action of these radiations as well as ultraviolet light has been reviewed by Lea (1947).

In addition to the lethal effect, ionizing radiations produce a delay in cell division, often with the production of elongated bacteria such as many chemotherapeutics produce. In contrast to the all-or-none lethal effect, this effect is graded in proportion to the dose of radiation to which the cell has been exposed. It is not known whether the effect is due to accumulation of poisons or destruction of metabolites; in either case metabolic processes eventually compensate for the change.

#### ULTRASONICS

Sound waves, which are longitudinal mechanical vibrations, are of course quite different in effect from the transverse electromagnetic vibrations of light. In the range exceeding the audible (called supersonic or ultrasonic), with a frequency of 15,000 to several hundred thousand per second, they coagulate protein solutions, disperse a variety of materials, and sterilize and disintegrate bacteria. Even audible sonic waves, in sufficient intensity, have been reported to be weakly bactericidal. The effect has not been of practical value as a means of sterilization, but has been found useful in extracting enzymes and antigens that are largely destroyed when bacteria are disintegrated by grinding.

#### FILTRATION

It is possible to obtain bacteria-free filtrates by the use of filters with a maximum pore size of  $1.5\ \mu$  or less. The Seitz filter, of asbestos, and the Berkefeld filter, of diatomaceous earth, are still widely used, but are quite adsorptive; in addition, the Seitz filter contributes considerable Ca and Fe to the solution, and therefore causes clotting of citrated plasma. The Chamberland unglazed porcelain filter has been modified with more accurate control of pore size by the modern porcelain industry, and effective sterilizing filters of sintered (fritted) glass (Corning UF) are also available. These two types of filters adsorb very little material and are ideal for the sterilization of sera, media which cannot be heated (e.g., media containing soluble proteins), and experimental materials in which possible effects of heating are to be avoided. They also permit quantitative recovery of bacteria for chemical or other purposes. The availability of such filters facilitates separate analysis of the changes that take place in the bacteria and in the medium during cultivation.

#### CHEMICAL AGENTS

##### GENERAL

Certain general statements can be made which apply to the action of all disinfectants. Bacterial species vary more widely in their susceptibility to disinfectants than to physical agents. This relative selectivity is of practical value in the preparation of selective diagnostic culture media. The action on a bacterial population is not instantaneous but is cumulative with time, and the rate increases with concentration and temperature, as is true of practically all chemical reactions.

Just as light quanta are effective only when absorbed, so disinfectants operate by chemical interaction with constituents of the bacteria. An exception is the possibility, raised by Dubos, that certain reversibly oxidizable dyes



are bacteriostatic by poisoning the redox potential of the medium at a value unfavorable for growth, i.e., interacting with constituents of the medium rather than the bacteria. The protoplasmic constituents with which the disinfectants combine are generally considered to be the proteins (including enzymes, constituents of membranes, and possibly constituents of genes); the nucleic acids, whose genetic importance has only recently been widely recognized, have not been sufficiently investigated. In a few cases, those of the detergents and the organic solvents, it seems possible that the effect involves interaction with lipid constituents of cell membranes.

Disinfectants are classified in pharmacology as "protoplasmic poisons," a vague term describing substances which depress the activity of all sorts of cells. Because this widespread effect implies a rather non-specific affinity for large classes of cellular constituents, it is not surprising that bacteria refuse to become appreciably adapted to these agents. This situation stands in marked contrast to the more selective action of the chemotherapeutic agents (including the antiseptic dyes), from whose inhibitory effects bacteria can frequently escape by developing suitable alternative metabolic pathways. It may also be remarked that whether a given substance is a meat or a poison to bacteria is often simply a matter of concentration: oxygen, salts, fatty acids, some vitamins and amino acids, and glycerol, in high enough concentrations, may be bacteriostatic and even actively bactericidal to bacteria which require them for growth.

Those antibacterial agents which are capable of ionizing as acids (anionic surface active agents including phenols; dyes; and organic acids including acidic chemotherapeutic agents) are more active with increasing acidity of the solution, while the opposite is true of cationic reagents. This effect depends upon either or both of two effects of the change in pH: increasing ionization of bacterial proteins in the direction opposite to the charge of the disinfectant, which increases the affinity, and decreasing ionization of the chemical agent, which increases

permeability. It is a well-established rule of cellular physiology that ions penetrate cell membranes much less readily than unionized molecules of comparable structure. The phenomenon can be analyzed as competition between the drug and  $H^+$  or  $OH^-$  ions.

Those disinfectants for which homologous series are available with varying aliphatic or aromatic hydrocarbon chains (e.g., alcohols, surface active agents) show an increase in bactericidal activity per unit concentration with increasing chain length; this parallels the increase in surface tension depression. Both effects show a maximum which varies from 8 to 10 carbon atoms for the alcohols and substituted phenols to 12 to 18 for the more soluble detergents.

The increasing size of the hydrophobic portion of the molecule tends to drive it out of aqueous solution, either to form an oriented layer at an air-water interface (surface tension effect) or to be absorbed on the surface of a bacterial cell membrane or protoplasmic macromolecule. The decreased activity and surface tension effect at chain lengths above the maximum is due to insufficient solubility. This relative insolubility is often inapparent and takes the special form of an increased tendency to aggregate in micelles which form colloidal solutions and are less surface active than the individual molecules. Disinfectants thus obey Traube's rule, which correlates the efficacy of narcotics and other pharmacologic agents with their effect upon surface tension. It would be an error, however, to regard this as the decisive factor, since many surface active compounds are ineffective; the specific architecture of the molecule, as emphasized by Ehrlich and Warburg, determines its avidity for essential macromolecular surfaces in the cell. When both properties are present they tend to be parallel, but a compound may be surface active without being antibacterial, e.g., phospholipids, or vice versa ( $HgCl_2$ ).

#### DISTILLED WATER

Distilled water is a less satisfactory medium than broth or other mixed media for the preservation of bacteria, although with many species it may be used as a temporary diluent without appreciable loss of

viability. It has been suggested that in some cases its toxic action is due to traces of heavy metals which would be ineffective in a diluent, such as broth, which contains organic materials that would compete with bacteria for the ions. The agents of water-borne infections, such as the typhoid bacillus, have been reported to survive for months in tap water. In general, bacteria differ strikingly from tissue cells in being quite indifferent to osmotic effects over a wide range. A few species, however, are lysed by distilled water, a phenomenon referred to as "plasmolysis."

#### ACIDS AND ALKALIS

Acids and alkalis are inhibitory to the growth of most bacteria outside a pH range of about 5 to 9. Fungi generally thrive at a more acid pH than bacteria. The range of some bacterial species is much more restricted than this. Indeed, W. M. Clark's classic studies on acid-base potentials and indicators arose from an investigation of the conditions of bacterial growth. With greater deviations from neutrality solutions become actively bactericidal, the disinfectant rates of various strong acids and bases being roughly correlated with the  $H^+$  or  $OH^-$  concentration. Acids tend to be more active than bases per unit concentration.

HCl or  $H_2SO_4$  completely disinfects a suspension of *E. coli* in 40 minutes in a concentration of the order of magnitude of 0.01 N. Mycobacteria, however, are somewhat more resistant to acid and alkali, it being common practice to liquefy sputum by exposure for 30 minutes to approximately 1 N. NaOH or  $H_2SO_4$  at 37° C. Gram-positive staphylococci and streptococci frequently survive this treatment too. Weak organic acids exert a greater effect than can be accounted for by the pH; this effect probably involves a specific affinity of the anion for cell proteins, but it may depend upon the greater permeability of cell membranes for unionized molecules. It is

the accumulation of organic acids, such as acetic or lactic, that limits the density of growth of many fermenting bacteria.

#### SALT

The practice of inhibiting bacterial growth by pickling in brine or by treatment with solid NaCl has been used for many centuries as a means of preserving perishable meats and fish. There is considerable variation in bacterial susceptibility; NaCl prevents the growth of tubercle bacilli at a concentration of 2 per cent, *E. coli* at around 6 per cent, and *B. subtilis* only at over 9 per cent. Certain organisms found in the Dead Sea, in brine, etc. require 10 to 12 per cent NaCl, and are called "halophilic."

Physiologic saline is not very suitable as a diluent for bacteria. Flexner observed that a trace of  $Ca^{++}$  prevented the disintegration of meningococci in this diluent; a balanced salt solution, therefore, appears to be desirable for some bacteria, just as Ringer long ago observed for heart muscle. A concentration of NaCl which makes water more bactericidal, however, may actually be beneficial in a nutrient medium.

#### METALLIC IONS

Mercury and silver form the most anti-bacterial salts,  $HgCl_2$  or  $AgNO_3$  preventing growth of many bacteria in concentrations less than 1 part per million, and killing small inocula in not very much higher concentrations. The various metallic ions can be arranged in a series of decreasing anti-bacterial activity, roughly the same for a variety of bacteria, with these two at the head of the list, then the other heavy metals, the iron group, the alkaline earths, and finally the alkali metals, of which Na has been described. Pharmacologists coined the term "oligodynamic action" to refer to the extremely high effective dilutions of metallic ions, the assumption being that the presence of comparatively few ions exerted



a remarkable effect on the cell. The effect is due, however, to the great affinity of certain proteins for these ions, which permits bacteria to take up relatively large amounts from very dilute solutions. It has been found that bacteria, trypanosomes, or yeast killed by Ag contain  $10^5$  to  $10^7$  Ag ions per cell, which is the same order of magnitude as the estimated number of enzyme protein molecules per cell (Clark, 1937). Because of this uptake the bactericidal concentration is markedly affected by the inoculum size and the presence of proteins in the medium.

It was mentioned earlier that the antibacterial action of Hg could be readily reversed by sulfhydryl compounds, whose affinity for Hg long ago gave rise to the term "mercaptan." There is good reason to believe that the biologic effect of this metal, as well as of arsenicals, depends upon similar combinations with sulfhydryl groups within the cell. The reversibility of the apparent bactericidal effect is readily understandable since a large number of enzymes have been shown to be reversibly inactivated by oxidation or other alteration (including combination with Hg) of their sulfhydryl groups. The action of heavy metals on bacteria is quite complicated, however, since they undoubtedly combine with a variety of cellular constituents and undergo redistribution after initial fixation, with eventual irreversible effects.

Mercuric chloride was long a popular disinfectant in 1:5,000 solution, though it is being largely replaced by organic surface active compounds which are more rapid and irreversible in action, less expensive and not so poisonous to man. The chemotherapeutic use of mercury or its salts in the treatment of syphilis is of historic interest only. A variety of organic mercury compounds, in which one of the valences of Hg is free to combine with protein, or else the Hg is united to the compound via a labile S or N linkage which permits slow dissociation of mercuric ion, are used as rela-

tively nonirritating antiseptics for skin and mucous membranes, and as antibacterial preservatives (e.g., merthiolate, mercurochrome, metaphen).

Silver has long been used as a relatively ineffective antiseptic in the form of a proteinate which slowly releases silver ions. The chemotherapeutic use of organic compounds of As, Bi, and Sb will be discussed later. The other metals have been of little value in bacteriology. Gold compounds have had temporary chemotherapeutic popularity. The tremendous importance in agriculture of copper salts as fungicides has found no parallel in medicine. Since most metallic surfaces become coated with oxides which contribute ions to solutions, the old tin cup may have been less of an epidemiologic hazard than the common drinking glass.

#### INORGANIC ANIONS

Inorganic anions are much less toxic than some of the cations. Boric acid has for some reason found wide use as an extremely mild antiseptic. Fluoride, which inhibits many enzymes, is toxic to many bacteria, but in concentrations far above those noted for Hg. Potassium tellurite, which is particularly inhibitory to Gram-negative organisms and least to corynebacteria, is used in the selective cultivation of diphtheria bacilli.

#### HALOGENS

Tincture of iodine (7 per cent or 3.5 per cent in alcohol containing KI) is one of the most rapidly acting bactericides. Although its painful and irritating effect on exposed tissue has led to its abandonment in the treatment of large wounds, it is an excellent antiseptic for skin and minor wounds.

Chlorine combines with water to form hypochlorous acid ( $\text{HOCl}$ ), an oxidizing agent which is rapidly bactericidal. Dakin's solution, freshly prepared hypochlorite of a standard strength, was widely used in World War I to irrigate wounds but has been re-

placed by modern chemotherapy. The azo-chloramides (e.g., Chloramine-T), organic compounds with a labile Cl atom attached to N, release free chlorine in solution and are less irritating to tissues. Chlorine in a dilution of a few parts per million is used to sterilize drinking water and swimming pools.

#### OXIDIZING AGENTS

Hydrogen peroxide ( $H_2O_2$ ), though marketed widely in a 3 per cent solution, cannot be strongly recommended as an antiseptic, even though patients derive satisfaction from seeing active bubbling when it is split by catalase in the tissues. Bacteria vary widely in their susceptibility, since some bacteria possess catalase; anaerobes in general lack this enzyme. Potassium permanganate ( $KMnO_4$ ) is of value as a urethral antiseptic in concentrations around 1/1,000 or less. These, as well as the halogens, presumably act by oxidizing and thereby inactivating enzymes (Knox et al., 1948).

#### FORMALDEHYDE

Formaldehyde ( $HCHO$ ) is marketed as a 37 per cent aqueous solution (formalin). It is particularly valuable as a means of sterilizing bacteria and inactivating bacterial toxins without destroying the antigenicity which is essential for their use in immunization. For this purpose exposure to a 1:1,000 solution for a number of hours is usually used. It is also used for urinary antisepsis by oral administration of a compound (methenamine) which releases formaldehyde in acid urine. Formaldehyde is capable of reacting with a variety of groups, especially  $NH_2$  and  $OH$  groups, which are abundant in proteins and nucleic acids. By replacing a hydrogen atom on two such groups on separate molecules, it forms methylene bridges which link the molecules permanently and it thereby hardens or fixes the tissue and inactivates most enzymes. There are, however, enzymes (e.g., verdo-

peroxidase) which are not inactivated by formaldehyde, and whose activity can be demonstrated histochemically on tissues fixed by formalin. Such a consideration helps us to understand the remarkable diversity of microbial life, which includes even molds and other micro-organisms capable of growing in formalin.

#### PHENOLS

Since Lister started spraying phenol (carbolic acid) through his surgical operating rooms, this compound has been considered the standard disinfectant. Actually, it is one of the least active of all the compounds mentioned in this chapter, and must be used in a concentration of over 1 per cent to produce reasonably rapid disinfection. Its activity is increased by chlorination, which increases the acidic dissociation of the phenolic  $OH$  group, or by substitution of alkyl groups on the benzene ring, which increase its surface activity and decrease its water solubility. Soap increases the activity of phenols up to a point, but if the concentration of soap is too high the phenol becomes largely distributed in the soap micelles and the disinfectant action drops to that of the soap alone. Probably the most widely used disinfectant for discarded bacteriologic material is a mixture of tricresol (mixed ortho, meta, and para methyl substituted phenol) and soap. In a sense the relatively weak action of phenol or cresol is of some advantage for this type of disinfection, since physicochemical characteristics which make it a weak germicide by virtue of a relatively weak affinity for proteins also make it much less susceptible than most other germicides to inhibition by the extraneous organic material present in discarded cultures, feces, etc.

Longer alkyl groups make phenols even more effective than cresol, but less soluble. Hexylresorcinol, phenol with a second  $OH$  group and a 6 carbon chain, is used as a skin antiseptic, and thymol (methyl iso-



propyl phenol) has such a low solubility that a crystal or two is a useful preservative of urine, enzymatic digests, etc.

Phenols are capable of inactivating enzymes, and Cooper has shown that in fairly high concentration they denature proteins, as shown by the fact that egg albumin so treated is insoluble at the isoelectric point even after removal of the phenol by dialysis. The bactericidal action of phenol has therefore been considered to involve denaturation of intracellular enzymes. Hotchkiss, however, has recently shown that phenol behaves like surface active agents in causing cytolysis. The evidence for this will be considered later; certainly the chemical structure of the phenols, with a hydrophilic, weakly ionizable group attached to a hydrophobic hydrocarbon, would permit their classification as weakly surface active agents. The relation between structure and activity of phenols has been reviewed by Suter (1941).

#### SOAPS AND OTHER SURFACE-ACTIVE AGENTS

Soaps (sodium or potassium salts of long chain fatty acids) have long been known to be bacteriostatic and bactericidal, especially to Gram-positive and acid-fast organisms. They visibly lyse pneumococci. Because of the relative resistance of Gram-negative species, oleic acid and, more recently, synthetic anionic soaps have been used for the selective cultivation of such organisms as *H. influenzae*. The use of soap in surgical scrubbing is partly bactericidal as well as detergent, but its selectivity prevents it from being a reliable general disinfectant. Lamar (1911), abetted by Flexner, suggested that fatty acids might participate in the natural defense of the body against bacteria; though no proof has been obtained in the many years since this suggestion, it cannot be dismissed lightly, since unknown defenses do exist, and fatty acids are almost the only known germicides which occur in

the body. They are chemotherapeutically ineffective by injection, however, because of their extraordinary affinity for serum albumin (Davis and Dubos, 1947). Chaulmoogric acid and other unsaturated fatty acids and esters in chaulmoogra oil have long been considered to have chemotherapeutic value in the treatment of leprosy, but the results have not been striking.

In recent years the investigation of this type of antibacterial compound has received a great impetus from the industrial development of a tremendous variety of synthetic detergents.

These are surface active compounds, i.e., compounds with a hydrophilic and a hydrophobic portion, which tend to accumulate in an oriented layer at aqueous interfaces with the hydrophobic region away from the water. They are commonly called detergents, although in many cases other surface active properties (wetting, emulsifying, foaming) are more prominent than their cleansing action. The nonionic detergents are not particularly antibacterial and in some cases are good bacterial nutrients; they will not be considered here. The anionic compounds generally have as their hydrophilic groups a sulfate ( $\text{RSO}_4\text{H}$ ) or sulfonate ( $\text{RSO}_3\text{H}$ ) group with highly variable R; phenols and carboxylates (fatty acids) are also anionic surface active compounds. The cationic compounds are usually substituted amines, ammonium, or heterocyclic nitrogenous compounds (e.g., pyridinium). The amines ionize only in acid solution; the most valuable compounds are the tetrasubstituted ammonium compounds ( $\text{R}_4\text{N}^+$ ), which are ionized at all pH's, and of these the best have a benzyl and a long chain group as well as two methyl groups (e.g., Zephiran, Phemerol). The tremendous variety of cationic germicides on the market is due to patent rights; they all have much the same action, some in greater dilution than others.

While the anionic detergents are relatively ineffective against Gram-negative organisms, the cationic compounds are practically equally effective against both Gram-positive and Gram-negative bacteria, and in 10 minutes will completely sterilize large

inocula of various species in dilutions of 1:3,000 to 1:30,000. They are consequently finding increasing use in a concentration of from 0.1 to 1.0 per cent as skin antiseptics, including surgical scrubbing, especially since they leave a fairly tenacious bactericidal surface film on the hands.

Considerably higher concentrations of cationic detergents are required to inactivate viruses than to kill bacteria; this property is useful in preventing bacterial contamination in preparation of vaccinia vaccine or in virus cultivation in eggs. These compounds are less toxic to tissues than many other germicides. Fuller observed bacteriostatic dilutions of over  $1:10^6$ , even in the presence of 50 per cent serum, for a number of compounds with an ionizable nitrogen (amine, ammonium, guanidine, amidine) at both ends of a long aliphatic chain. Nevertheless they did not prove to be antibacterial chemotherapeutics at permissible doses, even though the diamidines of this group,  $\text{H}_2\text{N}(\text{HN})\text{C}(\text{CH}_2)_n\text{C}(\text{NH})\text{NH}_2$ , were found by King to be effective in treating trypanosomiasis.

As might be expected from their configuration, cationic detergents are antagonized by anionic detergents and phospholipids, which combine with them by virtue of an opposite charge and a similar hydrophobic group (Baker et al., 1941a). Both anionic and cationic detergents denature proteins, and combine with them in amounts equivalent to 1/5 to 1/1 of the protein weight. It was generally considered that their mode of action, like that of most of the other disinfectants described above, depended upon inactivation of cellular enzymes. Hotchkiss (1946), however, has shown that they cause partial cytolysis in the same high dilutions that sterilize. This cytolysis is demonstrated by the extraction of cellular N and P by detergents (including phenol and soaps), but not following sterilization by metals, formaldehyde or halogens. It appears, then, that the primary action involves disorganization of the cell membrane, which may explain why they are less active against the less highly organized virus particles.

The antibacterial action of detergents is reviewed by Baker et al. (1941b) and Valko (1946).

#### ALCOHOL AND OTHER ORGANIC SOLVENTS

The disinfectant action of the aliphatic alcohols increases with chain length up to 8 to 10 carbon atoms, but the solubility in water decreases. Although ethyl alcohol has long received widest use, isopropyl alcohol, which is now available at low cost, has several advantages, being equally miscible with water, less volatile, more active, and not subject to legal restrictions as a potential beverage. The disinfectant action of alcohol, like its denaturing effect on soluble proteins, involves the participation of water. Ethyl alcohol is most effective in 50 to 70 per cent solution; 100 per cent alcohol is a poor disinfectant, in which anthrax spores have been reported to survive for as much as 50 days. Solutions of organic disinfectants such as formaldehyde or phenol are less effective in alcohol than in water, an indication of a lowered affinity of the disinfectant for the bacteria compared with its affinity for the solvent. While the bactericidal effect of ethyl alcohol is negligible at concentrations below 10 to 20 per cent, it is bacteriostatic for many organisms at concentrations as low as 1 per cent. The yeasts of alcoholic fermentation, however, can accumulate alcohol in the medium in concentrations up to 12 per cent or more.

While bacteria do not flourish in organic solvents, such as ether or benzene, these compounds are fairly unreliable as rapid disinfectants because of their low solubility in water. Protein solutions, enzymic digests, etc. are frequently saturated with toluene or chloroform to prevent mold growth. Glycerol, a polyhydric alcohol, is bacteriostatic in concentrations exceeding 50 per cent, even though it is an excellent nutrient in lower concentrations for many bacteria; it is used as a preservative diluent for vac-



TABLE 52. BACTERICIDAL CONCENTRATIONS (PROLONGED INCUBATION) \*

SUBSTANCE	BACTERICIDAL CONCENTRATION			
	S. AUREUS (GRAM POS.)		E. COLI (GRAM NEG.)	
	PEPTONE WATER	OX SERUM (HEATED 56°)	PEPTONE WATER	OX SERUM (HEATED 56°)
Acridine (acridine dye).....	1:200,000	1:200,000	1:20,000	1:100,000
Anil 48 (quinoline dye).....	1:200,000	1:200,000	1:200,000	1:200,000
Brilliant green (basic triphenyl methane dye)...	1:10,000,000	1:20,000	1:130,000	1:3,500
Mercurochrome (Hg-fluorescein dye).....	1:100,000	1:1,000	1:100,000	1:1,000
HgCl <sub>2</sub> .....	1:1,000,000	1:10,000	1:1,000,000	1:10,000
Phenol.....	1:250	1:250	1:250	1:250
Chloramine-T.....	1:2,000	1:250	1:2,000	1:250
Iodine.....	1:10,000	1:700	1:5,000	1:800

1 ml. samples of medium containing varying concentration of the disinfectants were given a small inoculum (0.1 ml. of a 1:1,000 or 1:20,000 dilution) of a 24 hour peptone water culture. The bactericidal concentration is the lowest concentration which, after incubation at 37° for 48 hours, prevented growth on subculture.

\* Modified from Browning, C. H.: Medical Research Council System of Bacteriology 1, 202 (1930); and British Medical Journal 1, 73 (1917).

cines and other biologicals since it is not irritating to tissues.

DYES

A wide variety of dyes have been shown to be bactericidal and bacteriostatic in dilutions, extending as high as 1:10<sup>7</sup> (Table 52). There is wide variation in the extent to which staining by various dyes is lethal. In general the basic dyes such as gentian violet are more effective, like the cationic detergents, than the acidic compounds. Undoubtedly the most valuable result of the large amount of systematic study of dyes was the discovery of sulfanilamide as an antibacterial breakdown-product of the dye Prontosil; the dyes themselves have proved of little value as antibacterial agents, except in selective cultivation. The main reason for this failure is probably their rapid adsorption onto plasma proteins and tissues, and the resulting poor penetration. Browning reported that the acridine dyes (often called "flavines," but not closely related to

the vitamin riboflavine) are not inactivated by serum, and recommended their use in wound antisepsis, but they are quite outmoded by modern chemotherapeutics. Albert et al. (1945) have admirably correlated the antibacterial action of acridine derivatives with their physicochemical properties.

AEROSOLS

The prevention of air-borne infection by sterilization of air is at present a lively field of investigation, and includes chemical disinfection as well as ultraviolet radiation. Robertson (1942, 1946) found that a number of glycols, such as propylene glycol and diethylene glycol, originally used as diluents for other disinfectants to be atomized as aerosols, are themselves powerful disinfectants when dispersed in fine droplets, even though they are only weak disinfectants in solution. They are effective in the air in high dilutions which are nontoxic to man. Their practical use, however, is limited by the fact that they are effective in only a narrow range of humidity.

Puck (1947) has demonstrated that the action of these chemical aerial disinfectants involves condensation of the glycol from the vapor phase onto the bacteria, rather than collision of bacteria with droplets. At high humidities the hygroscopic glycols have too low a vapor pressure owing to dilution by water taken up by their droplets; at low humidities the bacteria are desiccated and no longer attract glycols. It may be noted that the term aerosol, denoting a liquid in air emulsion, must be distinguished from the same word used as the trade name for certain anionic detergents.

#### GASEOUS DISINFECTANTS

Gaseous sulfur dioxide, chlorine, or formaldehyde may be used to disinfect articles of clothing, etc., which cannot be soaked or subjected to sterilizing temperatures.

### DYNAMICS OF STERILIZATION

#### TIME-ACTION CURVES

When bacteria are killed by ultraviolet or by ionizing radiations, the rate of killing follows an exponential (logarithmic) curve:  $n/n_0 = e^{-kt}$ , i.e.,  $\log n_0 - \log n = kt$ , where  $n_0$  is the initial number of viable bacteria,  $n$  is the number after exposure for time  $t$ , and  $k$  is a constant which depends upon the intensity of exposure. When the logarithm of the number of surviving bacteria is plotted against time, the data fall on a straight line. This means that the rate of sterilization is proportional to the number of survivors, and hence that the chance that a given cell will be sterilized during a given interval of exposure is independent of the duration of preceding exposure, i.e., the radiation exerts no cumulative effect. This is not surprising in the light of our knowledge of photochemistry. It is interpreted according to the "target" theory as meaning simply that death is caused by the irreversible effect of the absorption of a single quantum (or ionizing electron) in an appropriate loca-

tion in the cell, while absorption elsewhere in the cell is without lethal effect.

The aggregate of the sensitive loci make up the "sensitive volume," a small fraction of the total bacterial cell volume. This may be considered to represent the total volume of the essential genes, i.e., the group of autoreproductive nucleoprotein macromolecules of which every one is essential for cell multiplication. Alteration of single ordinary enzyme molecules by radiation is not lethal since each enzyme species is represented by a large number of individual molecules, and inactivation of an unknown, but probably large, proportion of the molecules of a given kind would be necessary to produce a lethal effect. Killing by this latter mechanism would involve a cumulative effect, and hence the curve would exhibit a plateau during the early stages of radiation. In practice, this effect does not occur to any significant effect with radiation since a lethal single hit occurs long before the lethal cumulative effect. A false sigmoid curve of this sort, with an initial plateau, is produced if the irradiated bacteria are clumped at the time of plating out for the quantitation of survivors, since the killing of the clump requires the accumulation of effective single hits on the individual cells. In this discussion it must be borne in mind that "killing" refers to destruction of the ability to propagate indefinitely and form a visible colony; irradiated spores may be quite alive enough to germinate even though the lethal effect prevents subsequent division.

While the above interpretation is generally accepted for the lethal action of radiation on bacteria, the same cannot be said for the exponential curves obtained with other agents. As early as 1908 Madsen and Nyman observed an exponential curve for the sterilization of bacteria by phenol, and a few years later Chick observed a similar curve for killing by heat, as also for coagulation of proteins by heat. There has been a large controversial literature concerning this "monomolecular" killing of bacteria, since the equation is that of a monomolecular chemical reaction. This does not mean that only one disinfectant molecule participates in the lethal reaction; but it does mean that the bacterial cell behaves in the



presence of a constant concentration of disinfectant as though the cell were a single unit, comparable to a single molecule, whose chance of undergoing a lethal reaction is independent of the duration of previous exposure.

The simplest physical picture for such a phenomenon would involve the reaction of one or more disinfectant molecules with a single indispensable bacterial molecule, of the nature of either a gene or an integral part of the cell membrane. This critical reaction might result in an alteration of avidity or permeability which would cause a large number of immediate subsequent reactions; but the lethal effect could still be defined as the single chemical "hit" that resulted in the altered threshold.

This interpretation involves the assumption that the individual cells in a bacterial population are uniformly susceptible to the action of heat or the disinfectant, and hence can be treated theoretically in a manner analogous to the statistical treatment of chemical reactions. Pharmacologists, who are accustomed to study the graded effect of various concentrations of chemicals on cellular functions or on the activity of enzyme solutions, are, in general, loath to accept bacterial death as an all-or-none effect on a single vital molecule in the cell. The alternative interpretation, which has been vigorously presented by Clark (1937), assumes that each individual cell has a definite length of survival under a given set of sterilizing conditions and that the gradual effect observed is an expression of the wide variation in susceptibility among the cells of a given population. This variation would have a very unusual, skewed distribution, with a maximal number of cells susceptible to an immediate effect of heat or the appropriate disinfectants, and progressively diminishing numbers susceptible to progressively longer exposure. This type of distribution, though unexpected, is not impossible; but this hypothesis is further weakened by the observation that the most perfect exponential disinfectant curves have been obtained with spores, which might be expected to vary less than vegetative cells.

Since the available data are consistent with either theory, and no critical experiment has been devised to choose between them, it is impossible to state where the truth lies. As our concept of genes acquires more physical and less purely formal character, however, the

"monomolecular" theory grows less implausible. It has been particularly advocated by Rahn (1945). It must be emphasized that exponential curves are not obtained with all chemical disinfectants; chlorine, for example, gives a sigmoid curve with an initial plateau.

#### CONCENTRATION-ACTION CURVES

The various concentrations of a disinfectant,  $c$ , required to sterilize a bacterial population in varying time,  $t$ , generally correspond quite closely to a curve which may be fitted by the equation  $c^n t = k$ . The concentration coefficient ( $n$ ) of most chemical agents has a value in the neighborhood of 1; in other words, the disinfecting time is inversely proportional to the concentration, over a wide range of concentration. Phenol, however, has the remarkably high concentration coefficient of 6 (Chart 15). For this

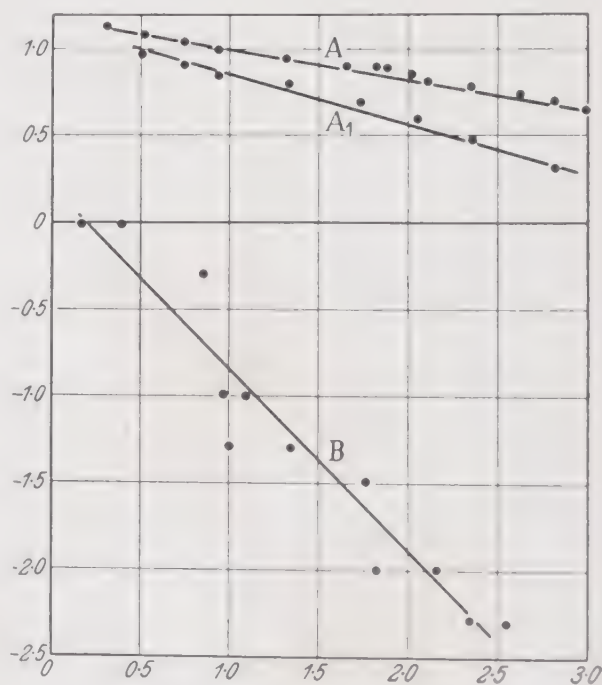


CHART 15. Time-concentration curves of disinfection of *S. paratyph*. Abscissa: log. time in minutes. Ordinate: log. concentration in per cent. (A) and (A<sub>1</sub>) phenol. (B) Mercuric chloride. From Clark (1937), p. 137, after Chick (1908).

Note the large shift in disinfection time caused by small shift in concentration of phenol.

reason it is particularly unfortunate that phenol is used as the standard of comparison in practically all the technics devised for the testing of disinfectants, from the early Rideal-Walker and the Chick to the modern FDA (Food and Drug Administration) test, which is widely used. The "phenol coefficient" furnished by such tests, i.e., the ratio of the concentration of phenol to the concentration of the tested compound necessary to sterilize a given suspension of *Staphylococcus aureus* or *E. typhosa*, can vary over an extremely wide range, up to

50-fold, depending upon the time chosen for the endpoint. It also varies widely with the test organism. A single value of the phenol coefficient of a compound gives very little information.

In practice it is necessary to test disinfectants under conditions (time, temperature, presence of fecal or other organic matter) which simulate as closely as possible the conditions under which they will have to be used. The practical aspects of disinfection and sterilization have been reviewed in detail by McCulloch (1936).

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## 35

# Principles of Chemotherapy

### GENERAL ASPECTS AND HISTORY

Although all therapeutic substances are chemicals, the word "chemotherapy" was firmly established by Ehrlich to denote the use of substances, other than antibodies, which inhibit or sterilize \* micro-organisms in the animal body. This category logically includes antimicrobial substances of biologic origin, as well as products of the organic chemist, for it is a matter of mere historic accident whether a particular compound is first extracted as a natural product (e.g., salicylic acid, quinine and the modern antibiotics) or is first isolated as a synthetic chemical. Substances which improve natural body defenses, however, such as vitamins or hormones (e.g., estrogens used in the treatment of gonococcal vulvovaginitis), are not considered chemotherapeutics.

Effective empirical chemotherapy was introduced to Europe in the seventeenth century with the importation of cinchona bark (containing quinine) and ipecacuanha root (containing emetine) from South America. But it was the phenomenal energy and imagination of Paul Ehrlich which applied the powerful tools of organic chemistry to the problems of infectious disease and created, in the first decade of this century, a well-developed science of chemotherapy: a science with not only a great deal of practical

success, but also a body of theory whose essential features are unlikely to be superseded. He demarcated this field from the rest of pharmacology in order to focus attention on the parasite rather than the host. But Ehrlich largely confined his attention to protozoal and spirochetal infections, for it seemed that in 1905 that bacterial infections were one by one succumbing to what he called the magic bullets (*Zauberkugeln*) of antibodies. Chemotherapy flourished—but not against bacteria.

For 30 years efforts by many workers to find a *therapia sterilisans magna* for bacterial diseases yielded at most a few dyes which found use as surface antiseptics. The initial promise against pneumococcus infections of ethylhydrocupreine ("optochin"), a quinine derivative, was not borne out clinically. But in the course of systematically testing on streptococcal infection in mice the newly synthesized dyes of the I. G. Farben industry in Germany, Domagk was able to report in 1935 the first effective chemotherapy of a systemic bacterial infection, using a red dye, Prontosil. Tréfouël and co-workers in France soon showed that the active agent was not the dye itself but a simple product of its hydrolysis, sulfanilamide. Since then bacteriology has truly entered the "golden age of chemotherapy." The rate of discovery is reminiscent of the earlier golden age, from 1880 to 1900, when

\* The reasons for preferring to speak of "sterilizing" rather than "killing" bacteria are stated in Chapter 34.

each month brought the discovery of a new etiologic agent, vaccine, or antiserum.

It must be emphasized that the central problem of chemotherapy is not that of discovering powerful antibacterial agents, i.e., those which act at very high dilution. The problem is the discovery of selectively cytotoxic compounds which inhibit pathogenic micro-organisms at concentrations in the body which can be tolerated by the host. The sulfonamides, for example, are therapeutically effective at concentrations in the body fluid of 2 to 10 mg. per cent, i.e., 1:50,000 to 1:10,000, while many of the disinfectants described in the preceding chapter are bacteriostatic in dilutions exceeding 1:1,000,000. Yet these "protoplasmic poisons" are far too toxic for use in the body. In addition, some of these compounds, especially the dyes and surface-active agents, have an affinity for various constituents of the blood that markedly limits their activity and distribution in the body.

Enthusiasm over mere *in vitro* antibacterial activity of a new microbial extract or new synthetic chemical is therefore premature, although under favorable circumstances testing in culture media has definite value in screening new products. Indeed, *in vitro* activity is neither a sufficient nor even a necessary condition of chemotherapeutic effectiveness. Had Prontosil not been tested in mice, its effectiveness, which depends on release of sulfanilamide in the body, would not have been revealed, and there would probably be little antibacterial chemotherapy to describe today.

At present chemotherapeutic research emphasizes antibiotics. Antibiotic relations between micro-organisms (i.e., the opposite of symbiosis) have been known since Pasteur and Joubert, in 1877, observed the death of anthrax bacilli in a contaminated culture. Attempts to use the substances responsible were made as early as 1899, when pyocyanase was partially purified from *B. pyocyaneus* by Emmerich and Low. It was assumed to be an enzyme but has since been

shown to consist of a complex mixture, including fatty acids. The term antibiotic, though perhaps unfortunate, was adapted by Waksman to refer to antibacterial substances of microbial origin. It carries no implication of the selectivity of action which is essential to chemotherapy; very few antibiotics have turned out to be chemotherapeutics. It appears reasonable to extend the term to products of plants and animals as well as microbes.

Two types of compounds frequently encountered in microbial extracts or culture filtrates seem particularly unlikely to have chemotherapeutic value: (1) fatty acids, whose limitations were discussed in the chapter on sterilization (p. 650), and (2) enzymes, which are antibacterial *in vitro* through the conversion of some component of the medium to a toxic product. Examples of the latter class are (a) penatin (notatin, penicillin B), which turned out to be glucose oxidase, producing hydrogen peroxide in the presence of glucose and oxygen, and (b) lipase, which frees fatty acids in a medium containing esters.

The mode of action of chemotherapeutics is still largely unknown. Ehrlich's views (1909, 1913), which are still stimulating (though severely criticized by Dale, 1923), are similar to his theory of immunologic reactions. They involve the concept that chemical groups on the drug match certain vital receptor groups in the micro-organism in the lock-and-key analogy derived from Emil Fischer's concept of enzyme specificity; the resulting combination interferes with the function of the cell. This view was not entirely speculative, but was based on the observation of decreased uptake of drugs by strains of trypanosomes with decreased sensitivity. The tremendous advance of biochemistry has as yet failed to give much insight into the nature of the hypothetical receptor groups, with the exceptions of the implication of sulfhydryl groups in the action of heavy metals, and a more concrete conception of the vital receptor molecules of protoplasm as enzymes and hence proteins.

A new pharmacologic principle has recently been added, as a result of work on the mode of action of sulfonamides which will be discussed later: the principle of competitive inhibition of a metabolic reaction by a compound which closely resembles in structure a normal metabolite (coenzyme or metabolic in-



intermediate) and hence can compete with the metabolite for an enzymic surface. This principle has received its greatest support through the prediction of the antibacterial activity of a variety of chemical analogues of known metabolites. It is too early to know how generally it applies to pharmacologic activity, but one may agree with Woolley (1946) that competitive inhibition probably represents a true current rather than an eddy in biochemical research. Nevertheless, the hope that this principle would elevate chemotherapeutic research from an empirical to a more rational, scientifically mature state has not been realized, for none of the metabolic analogues yet synthesized has been of practical chemotherapeutic value.

The history of chemotherapy, then, is one of a series of empirical trials, guided only to a limited extent by rational principles. Pron-tosil was the product of trial-and-error testing of a series of dyes manufactured for other purposes. The more active and less toxic sulfonamides which were subsequently developed by substitutions in the molecule, on the other hand, represent the greatest success of this more systematic approach, first introduced by Ehrlich with the arsenicals. Fleming's discovery of penicillin in 1929 was quite accidental. When 12 years later Chain and Florey undertook the task of purifying the labile active principle no one could have predicted that it would be the most valuable drug ever discovered rather than a toxic material. Since then systematic study of products of antibiotic micro-organisms has yielded a variety of antibacterial substances, but the vast majority of those studied up to now have been chemotherapeutically worthless because of toxicity or inactivity in the body. Probably the most rationally developed chemotherapeutic of all was tyrothricin (a mixture of gramicidin and tyrocidine), obtained by Dubos in 1939 from soil bacteria adapted to utilize Gram-positive bacteria as nutrients; but these products turned out to be too toxic for use in systemic infections. In spite of efforts to rationalize the field, it appears that the important discoveries of the near future are quite likely to be as empirically or accidentally achieved as those of the past. When the nature of the cellular receptors is known, chemotherapeutic research will develop a more physicochemical complexion, and will be integrated with the rest of biochemistry.

Successful chemotherapy has been limited to compounds that inhibit bacterial multipli-

cation. There are indications, however, that other approaches are also possible. Dubos and Avery adapted soil bacilli to grow on a substrate of the type-specific polysaccharide which is responsible for the virulence of pneumococci, and then purified polysaccharidase obtained from the adapted organisms. This enzyme protected animals against the corresponding type of pneumococci. Another approach is the antitoxic chemotherapy suggested by Zamecnik's recent observation that some protection can be furnished against the  $\alpha$  toxin of *B. welchii*, which is a lecithinase, by injecting lecithin to compete as a substrate with the host's tissue lecithin.

Although the number of useful chemotherapeutics is small, the search for more has become a big business. Students of a previous generation were inspired by Ehrlich's persistence in testing 606 arsenicals before discovering the useful compound arsphenamine. But by 1945 chemotherapeutic tests had been reported, largely by the pharmaceutical industry, for over 5,000 sulfonamides or sulfones, 10,000 arsenicals, and an even larger number of potential antimalarials; the variety of microbial extracts tested for antibiotic activity is undoubtedly of a similar order of magnitude. The literature of chemotherapy is tremendous and rapidly obsolescent. Because the practical details are constantly being improved, this chapter will emphasize the principles of application of chemotherapeutics and the little that is known of their mode of action on bacteria.

## METHODS OF TESTING

### IN VITRO

The synthetic chemotherapeutics which the chemist furnishes to the bacteriologist are relatively pure and need not be standardized biologically. In addition, chemical methods are available for estimating distribution in the tissues. Bacteriologic testing is therefore used only for determining the concentration of these drugs necessary to inhibit the growth of various species and strains of organisms.

Antibiotic research, however, must employ bacteriologic assay, with a test organism of fixed sensitivity, for a number of additional purposes as well: (1) estimation

of the activity of various fractions in the course of purification; (2) standardization of the purified product before use *in vivo*; and (3) assay of concentration in body fluids. Concentration is therefore expressed at first in terms of arbitrary units of bacteriostatic activity. When an antibiotic is eventually available as a pure product, the biologic unit is translated into terms of the fundamental unit of chemistry, mass. The history of antibiotic research thus parallels the standardization of other products with specific biologic activities, such as vitamins and hormones. It must be remembered, however, that crystallinity is not adequate evidence of chemical purity: the original crystalline penicillin turned out to be a mixture of closely related compounds with somewhat different biologic activities.

There are two general methods of estimating the bacteriostatic effectiveness of chemotherapeutics: the serial dilution method, and the so-called agar diffusion or cylinder plate method, first introduced for qualitative purposes, but later adapted to rapid quantitative estimation of antibiotics. In the serial dilution method a series of tubes of a liquid culture medium receive varying amounts of the drug in rather closely graded steps, and are then inoculated with a given number of organisms of a standard strain. The bacteriostatic concentration is taken as the minimum concentration which prevents either visible growth or production of a readily detectable product (such as hemolysin) after a given period of incubation. A sharper titration is often obtained by measuring the growth turbidimetrically and interpolating the value which would produce 50 per cent or 90 per cent inhibition of growth. The agar diffusion method depends on the concentration gradient of the drug diffusing through the agar from a central deposit in a bottomless glass cylinder or a porous porcelain cup imbedded in the medium. For quantitative work the medium is heavily and uniformly seeded with the test organism before pouring the plate, resulting in a continuous sheet of bacterial growth. The concentration of antibiotic is measured by the diameter of the clear area surrounding the cup, compared with the diameter surrounding known concentrations in other cups in the same agar plate. The agar-diffusion method involves less labor

but appears, on a-priori grounds, to furnish a less definitive answer than the serial dilution method, since the bacteria are exposed to drug concentrations which change progressively with time.

#### FACTORS AFFECTING ACTIVITY IN VITRO

**Sensitivity of the Bacteria.** Strains of many species as encountered in nature vary quite widely in sensitivity, in addition to developing increasing resistance following cultivation in the presence of the bacteriostatic agent. Among the antibiotics this variation is especially marked only in staphylococci and meningococci in relation to penicillin, but in most bacterial species in relation to streptomycin. Because of the possibility of alteration of this property in the course of numerous transfers, the standard strain is best preserved in the desiccated or frozen state.

**pH.** It was pointed out in connection with disinfectants that acidic compounds become more active with decreasing pH, and basic compounds with increasing pH. The same rule applies to the antibacterial chemotherapeutics. Sulfonamides dissociate as acids in the physiologic range of pH, but they also have a free amino group; they show a very marked decrease of activity at pH 6 to 7 compared with 7 to 9.

**Duration of Incubation.** Most chemotherapeutics markedly increase the lag period when used in concentrations too low to suppress growth permanently. In consequence, the minimum bacteriostatic concentration observed at 4 or 5 days may be several times as great as that observed at 1 day.

**Binding by Components of the Medium.** A wide variety of compounds, including sulfonamides and penicillins, are bound to a certain extent by serum albumin, and hence are less bacteriostatic in its presence. Phosphatides exert the same effect on surface active agents, including the antibiotic tyrocidine. Since chemotherapeutics are distributed in the body by the blood, it is essential that in-vitro testing of new agents include the use of media containing blood, serum or albumin. A good deal of confusion in the use of penicillin resulted from the practice of standardizing in the absence of serum. It was found that penicillin was a mixture of similar compounds, and that certain clinically disappointing lots contained an unusually high proportion of penicillin K. The explanation for its lower clinical activity was furnished by the



observation that penicillin K, which has a longer hydrophobic chain than penicillin X, G, or F, has a greater affinity for albumin (Tompsett et al., 1947). It is equally active per unit weight in a simple medium but is less active *in vivo*. The difference would have been detected by *in-vitro* tests in the presence of 3 to 5 per cent serum albumin.

In the bioassay of the concentration in the blood of a compound such as the penicillins, known to be partly bound to albumin, it is important to maintain an essentially constant concentration of serum albumin in the tubes in which the blood sample is serially diluted, as well as in the dilutions of the standard. Otherwise, with each dilution of the protein a portion of the reversibly bound drug becomes dissociated, and hence active. It is therefore not sufficient to add serum to the standard tube and then dilute both this and the unknown serum with medium, for the end points will generally occur at tubes in the two series having different protein concentration, and only apparently equal drug concentration.

Streptomycin is not significantly bound by serum.

**Metabolic Antagonists in the Medium.** Sulfonamides are antagonized not only by *p*-amino benzoic acid, but also to a smaller but significant extent by a variety of other compounds, including purines and unknown constituents of peptone. To avoid such interfering factors, experimental work on sulfonamide action has generally been carried out when possible in simple synthetic media. Streptomycin has been reported to be antagonized by very low concentrations of the phosphatide, lipositol, which may account for its decreased activity in media containing brain extract (Rhymer et al., 1947). Antagonists are not known for penicillin.

**Impurity of the Drug.** Antibiotic culture filtrates and cruder fractions often contain unidentified antibacterial agents in addition to that finally isolated; crude penicillin is reported to be more active per unit weight than the crystalline product.

**Destruction of the Drug.** Most chemotherapeutics are quite stable. Penicillin, however, is appreciably decomposed during even 24 hours of incubation. A few penicillin-resistant organisms produce an enzyme, penicillinase, which rapidly destroys the compound.

Blood culture from patients receiving chemotherapy is made more reliable by overcoming the effect of the drug present in

the blood. Para-amino benzoic acid is used to antagonize sulfonamides, and a bacterial extract rich in penicillinase to destroy penicillin. Such procedures are unnecessary, however, if the sample is diluted in a large enough volume of medium.

The technics of bioassay described above measure the bacteriostatic activity of chemotherapeutics. The bactericidal activity, which is less frequently measured, may be more significant for the mode of action. It can be determined simply as with disinfectants (Chapter 34): the organisms are exposed to the drug for a fixed period, diluted sufficiently to eliminate further drug action, subcultured on solid media, and compared quantitatively with control cultures.

### IN VIVO

We have emphasized that successful tests in animals are essential before a new extract or compound can be considered a promising chemotherapeutic. The technics are the usual statistical pharmacologic procedures for determining acute and chronic toxicity and the therapeutic index (the ratio of the lethal dose to the effective dose). Since modern chemotherapeutic research has emphasized the importance of maintaining a fairly constant concentration of drugs in the body (although this principle is now questioned), the therapeutic index is determined with a schedule of frequent doses, such as every four hours. In testing a new product the usual procedure, after determining *in-vitro* effectiveness when possible, is to determine the minimal lethal dose in normal animals and then test the effect of the largest tolerated dose, such as 50 to 80 per cent of the lethal dose, in infected animals. If this shows any promise, smaller, less toxic doses are studied. Since chemotherapeutics are expected to overcome clinical infections, it is important to test not only their prophylactic effect in animals infected at the time of starting chemotherapy, but also their effect on well-established

lished infections. The test of a drug and a bacterial inoculum simultaneously injected into the same body cavity is not really a test of chemotherapy at all.

The effect of a drug on tissue cultures, or on motility of leukocytes, is sometimes used as an index of toxicity to host cells.

Clinical effectiveness and toxicity must ultimately be determined in patients; the type of toxicity which limits dosage in man is ordinarily not the lethal toxicity tested in animals. Nevertheless, it is generally true that animal experiments have given a very useful prediction of the clinical behavior of chemotherapeutics. Tuberculosis is something of an exception, since the sulfones and streptomycin have appeared more promising when tested on tuberculous guinea pigs than when tested on the more caseous, destructive, and fibroid type of disease generally encountered in man.

## MODE OF ACTION

With few exceptions (Prontosil, some arsenicals) the chemotherapeutics act on bacteria *in vivo* in the form administered, without requiring conversion by the body to an active compound. The action on bacteria is direct and does not require the participation of host mechanisms, except for the important subsequent step of eliminating bacteria which have been inhibited but not sterilized. Although the action of drugs on cells is properly the subject of pharmacology, it seems appropriate to discuss here some of the interesting effects on bacterial cells which have been disclosed by extensive recent investigation in this field (Faraday Society, 1943).

## PHARMACODYNAMIC GROUPS

The similar pharmacologic action of related compounds can be ascribed primarily to the effect of their common structural features. The structure common to all the active sulfonamides and sulfones is a free aromatic amino group para to the sulfonic group. Acetylation of the amino group

removes all antibacterial activity. When more effective derivatives of sulfanilamide were first synthesized, it was noted that the increasing activity, from sulfanilamide to sulfapyridine and then sulfadiazine and sulfathiazole, was paralleled by increasing acidic dissociation of the sulfonamide group (i.e., decreasing  $pK$ , from 10 to 6.8); in other words, the higher the proportion of ionized molecules at the pH of the body or the culture, the greater the antibacterial activity. With a given compound the activity was decreased by lowering the pH, which decreased the dissociation of the compound.

Bell and Roblin (1942), however, studied a much more extensive series of compounds and found that with further decrease in the  $pK$  (e.g., sulfacetamide) there was a reversal of the effect, antibacterial activity being decreased. Sulfonamides with a  $pK$  of about 6.7 had maximal activity. Ionization is therefore not the variable fundamental to antibacterial action.

They noted that a physicochemical parameter which goes through the same maximum, and follows quite closely the distribution of antibacterial activity in the series of compounds, is the negativity of the  $SO_2$  group, i.e., the tendency of this group to attract electrons from other atoms in the molecule. The parallelism of ionization and activity therefore appears to be a coincidental relationship, valid over only the limited range of weakly dissociated compounds, among which  $SO_2$  negativity increases with increasing ionization. But with more strongly dissociated compounds (lower  $pK$ ) most of the molecules are ionized and bear a formal negative charge; there are few unionized molecules remaining to display the type of negativity which appears to be significant here. To the results of Bell and Roblin may be added the observation of Kumler and Daniels (1943) that this negativity is associated with an attraction of electrons from the opposite free amino group (which would be suppressed by acetylation), and hence an increased dipolar structure of the molecule.

This work is the most profound physicochemical analysis of a pharmacodynamic group yet produced, and casts doubt on the classic



association of the activity of other pharmacodynamic groups with their ionization. In making such correlations it must be remembered that series of related compounds generally have several physicochemical properties varying in the same direction, and hence that parallelism of pharmacologic activity with surface tension, lipid solubility, dissociation constant or, in this case, negativity of a group, may not furnish the key to the mechanism of pharmacological action—which, in the last analysis, most likely depends on specific affinity for a cellular constituent.

The nature of the cellular receptor groups for the sulfonamides is unknown, but the author has shown, for a smaller series of compounds than those studied by Bell and Roblin, that affinity for serum albumin runs parallel to antibacterial activity. This suggests that the nature of the regions on the protein surface responsible for the binding may give a clue to the nature of the corresponding regions in the cell.

Arsenicals appear to act by virtue of the affinity of the trivalent arsenoxide group for sulphydryl groups, which are present on many enzymes. This was originally suggested by Ehrlich, and demonstrated by Voegtlin for glutathione in 1925, and by Rosenthal for sulphydryl groups of tissue proteins. During World War II a compound called BAL (British Anti-Lewisite,  $\text{CH}_2\text{OH}\cdot\text{CHSH}\cdot\text{CH}_2\text{SH}$ ) was prepared as an antidote for an arsenical poison gas, and proved to be of value in treating toxic reactions to arsenical drugs and mercury poisoning. It is effective by virtue of having two sulphydryl groups on adjacent carbon atoms, which provide a greater affinity for the metals than do isolated sulphydryl groups. Though this compound is not an antibacterial chemotherapeutic, it is mentioned here as an example of the power of chemistry to serve pharmacology when the nature of the interacting groups is known.

#### COMPETITIVE INHIBITION

Competitive inhibition of an enzyme, by a compound structurally related to the normal substrate, had been demonstrated

at least 20 years ago by Quastel, with the inhibition of succinic dehydrogenase by malonic acid. This compound, which is one carbon atom shorter than the substrate succinic acid, presumably competes with the substrate for the same position on the enzyme molecule but remains fixed there without undergoing oxidation. This first became an important pharmacologic concept, extended to the functions of a cell rather than an enzyme, in the classic paper of D. D. Woods (1940). He demonstrated indirectly, by testing the effect of a variety of reagents on yeast extract, that the substance responsible for the antagonism of the extract for sulfanilamide had the properties of p-amino benzoic acid (PABA). This compound was found to antagonize sulfanilamide in amazingly low concentrations, as low as 1/26,000 that of the drug. The antagonism satisfied the criterion of a competitive reaction—that is to say, over quite a range of concentration the threshold of antagonism appeared at a constant ratio of antagonist to bacterial inhibitor. Noncompetitive inhibitors of enzymic or cellular function are not displaced in this manner by excess of substrate. When much more powerful sulfonamides were developed, which, like PABA, were to a considerable extent ionized at the pH of testing, the competitive ratio of PABA to sulfonamide was found to be much higher, and to approach a value of 1/1. The interpretation given to the phenomenon by Woods and by Fildes (1940) was that PABA participates in some unknown essential metabolic reaction in the cell and that sulfonamides attach themselves to the enzyme involved and thereby prevent access of PABA. The affinity of PABA and sulfonamides for the same active sites on enzyme molecules is presumed to be due to their marked structural similarity, sulfanilamide being PABA in which an acidic  $\text{SO}_2\text{NH}_2$  group has been substituted for the acidic  $\text{CO}_2\text{H}$  group (p. 676). The competition is analogous to that of carbon monoxide and oxygen for hemoglobin, the proportion

of the two gases combined depending on their relative partial pressures and on the dissociation constants of oxyhemoglobin and carboxyhemoglobin.

Widespread investigation of the possible role of PABA as an essential nutrient revealed only a few organisms which require traces of PABA in the medium; these include strains of the nonpathogenic *Cl. acetobutylicum* and *Acetobacter suboxydans*. It is consequently necessary to assume, in order to maintain the Woods-Fildes hypothesis, that most bacteria synthesize the PABA necessary for their normal metabolism, just as they synthesize amino acids or other essential components of the cell.

Efforts to find an enzyme system involving PABA as coenzyme failed. Recently, however, PABA was found to be a component of the vitamin folic acid (pteroyl glutamic acid); the PABA is linked to a nitrogenous base, a pterin (which is intermediate in structure between a pyrimidine and a flavine). The resulting pteric acid is conjugated with one or more molecules of glutamic acid. By analogy with the coenzymic role of riboflavine, thiamine and other vitamins which are universally distributed in bacterial and animal cells, it seems exceedingly likely that pteroyl glutamic acid is a coenzyme whose function is as yet undiscovered. The sketch is now beginning to be filled in, though the brushwork is not complete. PABA is a cog in a piece of metabolic apparatus; sulfonamides prevent PABA from being synthesized into the working machine, either to replace losses or to satisfy growth requirements. This scheme explains why sulfonamides do not halt cell division at once, since the cells can manage a few divisions on the normal store of PABA derivatives. It also explains the noncompetitive weak antagonism of sulfonamides by a variety of compounds (purines, methionine) if it is assumed that the PABA derivatives are concerned with reactions involved in the synthesis of these compounds. But folic acid antagonizes sulfonamides only in the case of a few organisms; presumably in others the PABA is synthesized into other derivatives (Lampen and Jones, 1946). The low toxicity for host cells may depend upon their lack of a sulfonamide-sensitive system for synthesizing the required folic acid.

No competitive inhibitors are known for penicillin or streptomycin. This fact does not conflict with the general belief that these drugs

likewise inhibit enzyme systems, for many known enzyme inhibitors, such as metal ions, are noncompetitive.

The concept of competitive inhibition has stimulated the synthesis of many analogues of known metabolites, such as amino acids, nicotinic acid, or pantothenic acid in which a carboxyl group is replaced by a sulfonic group, or analogues of thiamine or biotin with altered ring structure (Welch, 1945; Woolley, 1946; Roblin, 1946). Although these compounds inhibit the growth of bacteria or animals, no practical chemotherapeutic has yet resulted from this work. One probable reason is the presence in the host of these known metabolites, which antagonize their analogues. McIlwain and Hawking have shown, for example, that pantoyl taurine (sulfonic substituted pantothenic acid) protects rats against streptococci, but fails in mice, which are known to have a higher blood concentration of pantothenic acid.

#### SUSCEPTIBLE SPECIES

The penicillins and gramicidin inhibit most Gram-positive bacterial species and the Gram-negative gonococcus and meningococcus, and penicillin inhibits spirochetes as well. Streptomycin inhibits most Gram-negative species and also the acid-fast mycobacteria. Sulfonamides inhibit a wider range of organisms, including members of all three bacterial groups and some large viruses. Each of the experimental antibiotics inhibits a characteristic group of susceptible organisms. This "antibacterial spectrum" (including the use of adapted drug-resistant strains) offers the earliest intimation of the identity of antibiotics from different sources, before isolation permits chemical comparison. This problem is very real: several antibiotics have appeared in the literature under two or three names before the identity of the products was realized.

Gram staining is of practical value in roughly summarizing some antibacterial



spectra. The conspicuous exception of the marked sensitivity of Gram-negative *Neisseria* to penicillin may not be too significant, since, as Dubos has emphasized, these organisms share several other characteristics with Gram-positive organisms and hence appear to present a borderline problem in classification. Nevertheless, the basis of the Gram stain, even if more fully understood, would not be likely to assist us in understanding the subtle mechanisms underlying chemotherapeutic action. The possibility of developing highly resistant variants from sensitive species, without alteration of staining properties, indicates that the susceptibility of bacteria to various inhibitors is not fundamentally connected with the structures responsible for Gram staining. Gram staining, therefore, appears to be associated with chemotherapeutic response only in a loose way, rather than through a direct causal connection. It may be pointed out, however, that when staphylococci were rendered extremely resistant to penicillin by successive selection, they lost their Gram-positive response to staining. (Klimek et al., 1948.)

It must be emphasized that the differences in susceptibility of bacterial species are only quantitative; the chemotherapeutics under discussion inhibit practically all bacteria *in vitro* in sufficient concentration, though different mechanisms may possibly be involved at high and low concentrations.

#### EFFECT ON METABOLISM AND CELL DIVISION

An important characteristic of the chemotherapeutics is their interaction with cellular metabolism. Streptomycin sterilizes at a high rate only in the presence of sufficient nutrient (such as glucose) and phosphate to permit active respiration or fermentation. Sulfonamides permit 6 to 8 cell divisions before exerting their bacteriostatic action on *E. coli*; this phenomenon presumably depends on the presence of accumulated PABA derivatives. Penicillin is even more

fastidious in its requirements: it sterilizes only under conditions of temperature and complete nutrition which would permit bacterial growth in the absence of the drug. It is clear that these drugs do not sterilize by simple denaturation of proteins. They are thereby distinguished from the disinfectants, which generally sterilize even more rapidly in saline or water than in a complete medium. This characteristic of the chemotherapeutics probably has great significance for their mode of action in the body, in which complete eradication of the bacteria is a much slower process than the initial overwhelming attack. Tyrocidine, which is an antibiotic but is too toxic to be an effective chemotherapeutic, also has a disinfectant mode of action, resembling the cationic detergents (Hotchkiss, 1944).

While it is clear that chemotherapeutics act by selectively influencing metabolic processes, there has been only one major advance in the analysis of the metabolic effects. This is the relation of PABA, and therefore sulfonamides, to pteroyl glutamic acid (folic acid) (page 663). We have no reliable clue to the primary effect of penicillin, except that its requirement of bacterial growth for its lethal effect indicates that it interacts irreversibly in some way with a process of synthesis that takes place only during active cellular growth. A striking biochemical effect of this attack is the loss of the normal ability of Gram-positive bacteria to accumulate a high intracellular concentration of amino acids (Gale and Taylor, 1947).

Streptomycin is antagonized by a variety of salts, and is very sensitive to the salt concentration of the medium; the bactericidal concentration is reduced a thousand-fold by eliminating from the medium all the salt but the trace of phosphate necessary for glycolysis. Since the drug is a highly charged cation, this effect suggests a non-specific competition with other cations. Streptomycin is also antagonized by lipositol, a phosphatide containing inositol, to

which a portion of the drug molecule is related; strictly competitive inhibition, however, as in the fruitful case of PABA and sulfanilamide, has not been shown (Rhymer et al., 1947). That streptomycin is intimately concerned with a metabolic process can be inferred from the remarkable observation of Miller and Bohnhoff (1947) that some strains of meningococcus which had developed resistance to streptomycin actually required this compound as a growth factor! This observation has been extended by others to a variety of species, including mycobacteria and *E. coli*. It has also been observed that streptomycin in subinhibitory amounts acts as a growth stimulant (though not necessarily a requirement) of some of the resistant strains. As this latter phenomenon has been observed in vitro with tubercle bacilli isolated from streptomycin-treated patients, it probably occurs in vivo as well—a matter of obvious clinical importance.

During the past two decades, since the development of the Warburg microrespirometer, the greatest success of biochemistry has been the analysis of the catabolic processes of respiration and fermentation which furnish free energy for the maintenance and growth of cell structures. But of the mechanism by which this energy is utilized for the anabolic processes of synthesis of protoplasm we know nothing, beyond the growing conviction that protein synthesis is not a mere reversal of hydrolysis under the influence of proteolytic enzymes. Because the technics of studying respiration were well developed, investigation of the mode of action of chemotherapeutics, especially sulfonamides, led to a great deal of work with Warburg vessels. There were numerous reports of inhibition of total cellular respiration and inhibition of the action of various isolated enzymes by chemotherapeutics. The inhibitory effect on the known enzymes, however, generally required much higher concentrations than did bacteriostasis. With regard to the inhibition of respiration (which was used as a device for screening potential antimalarials, for example), it is necessary to point out that gramicidin, streptomycin and p-amino salicylic acid, at bacteriostatic concentrations, cause a marked increase

in bacterial respiration of certain organisms for several hours, followed by a decrease when the cellular organization has sufficiently disintegrated. It is evident that normal respiration is not necessarily the highest possible respiration, and that enzyme inhibitors and enzyme competition for substrates are important aspects of an integrated cellular economy, along with enzyme activity.

Since the chemotherapeutics do not appear to act via the catabolic enzymes with which we are familiar, it appears that their effect is exerted somewhere in the much larger unknown mass of enzymes responsible for synthetic reactions. This is not a surprising conclusion, as the inhibition of bacterial growth (i.e., total synthesis) is the common characteristic of these drugs. The results reported thus far on metabolic investigation of the mode of action of chemotherapeutics must be judged with caution. To explore this important territory, it appears that chemotherapy would profit by, and may contribute to, the study of cellular synthetic reactions. For a more extended discussion of chemotherapy as applied to cytochemistry, the reader is referred to Hotchkiss (1946).

Most chemotherapeutics, and especially penicillin, produce a peculiar inhibition of bacterial cell division in concentrations which only partly inhibit growth. The resulting cells are enormous, with elongated bacilli sometimes extending across an entire microscopic field. The effect is too nonspecific to be illuminating, however, as a similar effect has been observed following ultraviolet irradiation or exposure to dyes. Certain species (*Salmonella coli*) show lysis of these enlarged cells, but the effect undoubtedly depends on the autolytic enzymes of the inhibited bacteria rather than a direct lytic effect of the drug.

#### QUANTITATIVE RELATIONS

It has been pointed out that the remarkably low effective concentration of some chemotherapeutics (such as penicillin, effective at 0.01 to 0.1  $\mu\text{g./ml.}$ ) is only a superficial characteristic. While the chemotherapeutics are distinguished in this manner from a variety of disinfectants such as alcohol or phenol, other disinfectants (Hg, Ag), and such toxic antibiotics as gramicidin and actinomycin A, are effective at equally high or higher dilutions. But pub-



lished tables comparing the effective concentrations of antibacterial compounds must be critically judged, since the bacteriostatic concentrations of chemotherapeutics are usually compared with the concentrations of disinfectants required for rapid sterilization.

It might be interesting to see if the effectiveness of chemotherapeutics is so remarkable when calculated in terms of the number of molecules available. A solution of 0.1  $\mu\text{g./ml.}$  of penicillin (molecular weight 320) contains  $3 \times 10^{-10}$  mols/ml.; since the number of molecules per mol (Avogadro's number) is  $6 \times 10^{23}$ , this solution contains  $1.8 \times 10^{14}$  molecules per ml. (This is three times the molar concentration of  $\text{H}^+$  or  $\text{OH}^-$  ions at neutrality.) A fair-sized coccus occupies a volume of 1 cubic micron, or  $10^{-12}$  ml. If the drug were uniformly distributed throughout the volume of bacteria and solution, each coccus would contain 180 molecules of penicillin. Actually, although no data are available on the uptake of penicillin by bacteria, it is extremely likely that the affinity of certain protoplasmic constituents for penicillin leads to selective absorption by the bacteria of much larger amounts of the drug, though not necessarily as extensive as has been shown for the less selective "oligodynamic" action of Ag or As. Because of the low ratio of bacterial to fluid volume in even a heavily septicemic blood (e.g., 100 bacteria per ml.), every cell could absorb thousands of molecules of penicillin from a bacteriostatic solution without producing a detectable decrease in concentration in the solution.

## FACTORS AFFECTING ACTIVITY IN THE BODY

### CONCENTRATION

The simplicity of the chemical analysis for sulfonamides led to extensive studies of their concentration in body fluids, unparalleled in the history of pharmacology, and to an emphasis on the relation between activity and concentration rather than activity and dosage. The most significant conclusions of these studies were: (1) the possibility of speeding therapy by giving a large initial "saturation" dose, to produce imme-

diately an effective concentration in the blood, and (2) the necessity of giving small maintenance doses at much more frequent intervals than had been recognized with earlier drugs if continuous effective concentrations were to be maintained in the blood. These concepts led to improvements in the mode of administering long known drugs, such as salicylates, antimalarials and arsenicals.

The importance of the earliest possible institution of chemotherapy in severe acute infections, such as pneumonia or meningitis, must be emphasized. Not only does a longer period of unchecked bacterial multiplication result in greater toxemia and tissue damage, but it must be recognized that chemotherapeutics do not entirely inhibit bacterial multiplication as soon as an effective blood concentration has been reached. The lag is due partly to the time necessary for penetration to the sites of infection, and in addition, in the case of the sulfonamides, to the fact that bacteriostasis appears only after several cell divisions in the presence of the drug. An overwhelming case of meningitis can thereby progress to a fatal end in the presence of adequate drug concentrations. For this reason, together with the low toxicity of modern chemotherapeutics (especially penicillin), the immediate institution of chemotherapy of severe acute infections on the basis of a presumptive clinical diagnosis is justified as soon as samples have been taken for bacteriological analysis. On the other hand, this view does not justify "shotgun" therapy without bacteriologic diagnosis. It must be emphasized that accurate bacteriologic diagnosis becomes increasingly important with the increasing multiplicity of available chemotherapeutics. For example, pneumococcus and Klebsiella pneumonias are clinically identical, but totally different in chemotherapeutic response.

The antibacterial chemotherapeutics are so rapidly excreted that continuous effective blood levels without excessive dosage can ordinarily be maintained only by adminis-

tration at intervals of 3 to 4 hours. With the oral administration of sulfonamides this causes no inconvenience. Such frequent injections of penicillin or streptomycin, however, are annoying to the patient and require much nursing or medical time. It has been found possible to delay the absorption of penicillin from the injection site, and hence prolong its action, by suspending the drug in beeswax and oil or by forming a relatively insoluble complex of the acidic drug with a base (e.g., procaine penicillin). This device permits ambulatory treatment of gonorrhea, for example, by one injection, with considerable saving in cost.

There is a growing belief today that the importance of continuous effective blood concentrations has been overemphasized. Equally satisfactory clinical results have been reported following treatment of a variety of infections with injections of penicillin or streptomycin at intervals of 8 to 24 hours rather than 3 or 4 hours. Such intermittent blood concentrations have more theoretical justification in the case of slowly multiplying organisms, such as the tubercle bacillus (with a mean generation time *in vitro* of 18 hours or more), than in the case of most bacteria of acute infections (with a mean generation time of 20 to 40 minutes). The matter still awaits solution. Intermittent therapy, if as effective as continuous therapy, is in most cases simply more convenient. Where toxicity limits total dosage, however, as in the streptomycin treatment of tuberculosis, intermittent therapy may have a fundamental advantage.

The recent reconsideration of the earlier assumed desirability of continuous blood concentrations (Marshall, 1947) has been very much influenced by the experience with anti-malarials, in which effective action could not be correlated with plasma levels. In addition, compounds such as quinacrine (atebrin), which are effective in an infrequent dosage schedule, accumulate in some tissues in concentrations as high as 1,000 times the plasma concentration; the tissues presumably slowly release the drug to maintain an effective concentration long after administration has ceased. The importance of this "factor of persistence" was early emphasized by Dale (1923) after its demonstration for arsenicals and for the trypanosomicide germanin (Bayer 205). It must be emphasized, however, that experience with

such persistent compounds cannot be transferred to the rapidly excreted antibacterial chemotherapeutics.

Nevertheless, experiments on animals and patients have shown that optimal therapeutic results with penicillin and streptomycin appear to be less dependent on continuous effective blood concentrations than is the case with sulfonamides. A theoretical explanation for this apparent difference can be offered. Sulfonamides are primarily, if not entirely, bacteriostatic *in vitro*, presumably relying on host defense mechanisms for final elimination of the bacteria *in vivo*. As these host mechanisms are relatively slow, periods of low drug concentration would permit multiplication to start from a large residue of previously inhibited but still viable bacteria. Penicillin and streptomycin, however, are rapidly bactericidal *in vitro*. Although many hours are required for them to sterilize a culture completely in ordinary concentrations, a very large proportion of the organisms succumb within a few hours. *In vivo*, it is difficult to test for this direct bactericidal action of a drug, since host mechanisms are also present, but there is little reason to doubt that the effect observed *in vitro* must also take place in the host. A single adequate dose *in vivo* would therefore sterilize most of the bacteria; subsequent multiplication during a drug-free period would be based on only a small viable residue, which would not reach large numbers before the next injection of the drug a number of hours later.

The advantage of intermittent effective levels has presumably been further supported by the argument that penicillin is bactericidal only to multiplying organisms, and hence intermittent freedom from the drug would increase susceptibility by permitting intermittent multiplication. This argument is not valid, since the available evidence shows merely that the drug is bactericidal only under conditions which would permit multiplication in the absence of the drug. This is a very different matter from supposing that the drug sterilizes only when it can "sneak up" on an unsuspecting bacterium in the act of fission.

The aim of most pharmacologic studies on chemotherapeutics has been to determine the most convenient mode of administration and dosage which will maintain an effective antibacterial blood level throughout the period of therapy. Not only is the importance of continuous maintenance now questioned, however, but several considerations support the



view that it is sometimes advantageous to produce higher concentrations than those required *in vitro*. (1) The antibacterial concentration determined *in vitro* is a "bacteriostatic" concentration, i.e., the minimal concentration which will prevent visible growth, either by true bacteriostasis or by slow bactericidal action. But bactericidal drugs sterilize more rapidly at higher concentrations. The bactericidal rate of streptomycin appears to increase more or less indefinitely with concentration. With penicillin, however, Eagle (1948) has shown that a maximal rate is obtained at roughly 10 times the minimal effective concentration; above this level the rate is essentially constant, and with some bacterial strains it even decreases at excessively high concentrations. (2) There is a time lag in establishing in the tissues the same concentration as is present in the blood; in relatively avascular areas of necrotic or granulomatous infected tissue this lag is probably quite long. Since the rate of diffusion of any substance from a source, such as a blood vessel, is directly proportional to the concentration present, it is obvious that an excessive concentration in the blood will hasten the establishment of an adequate concentration in the infected tissues. (3) During the course of treatment moderately drug-resistant organisms may appear, which would be susceptible only to higher than minimal levels.

"Rational" chemotherapy is therefore based on an oversimplified rationale if it relies exclusively on maintaining an ideal minimal effective blood concentration. On the other hand, the above considerations do not justify the practice of taking advantage of the low toxicity of penicillin to give "for good measure" as much as millions of units a day, producing blood levels of over 100 times the minimal inhibitory concentration. The decision concerning the advantage of high dosage in certain situations, or of intermittent rather than continuous therapy, must ultimately be based on empirical clinical observations.

#### DISTRIBUTION; BINDING BY PROTEINS

The interaction of chemotherapeutics with serum albumin, noted above in con-

nection with *in-vitro* testing, is an important factor in determining their distribution and effectiveness in the body. The binding is practically instantaneous and reversible. In a patient receiving a sulfonamide the drug molecules exist in the plasma in the following forms: (1) conjugated (chiefly acetylated), which has been irreversibly inactivated in the body, and is not included in the usual chemical determination of blood level; (2) bound to albumin, which is inactive but forms a reservoir of potentially active drug; and (3) unbound, which is the active drug. This last fraction may be further divided into an ionized and an unionized portion, but the relation of ionization to activity is not a simple one. As the concentration of unbound drug in the circulation is decreased by conjugation or excretion, a portion of the bound drug is dissociated to establish a new equilibrium. Sulfathiazole is approximately 80 per cent bound in normal plasma, sulfadiazine 50 per cent and sulfanilamide 20 per cent (Davis, 1943).

The determination of bound and unbound drug requires dialysis; the usual chemical estimation does not distinguish the two, for it involves preparation of a protein-free blood filtrate at an acid pH at which the drug-protein complex is completely dissociated.

Cerebrospinal fluid and several other body fluids behave as ultrafiltrates of plasma in respect to the commonly used sulfonamides; after sufficient time has elapsed to permit equilibration, the total concentration in the practically protein-free CSF is essentially equal to the unbound concentration of the plasma. It was thought at first that sulfanilamide was the drug of choice in treating meningitis, since it was the only one to produce CSF concentrations nearly as high as the blood levels. A CSF sulfathiazole concentration of 1 mg. per cent, however, is as bacteriostatic as the corresponding 5 mg. per cent in the plasma, of which only 1 mg. per cent is active. Since those sulfonamides with a higher affinity for albumin

also have a markedly greater antibacterial activity, which more than compensates for the inhibiting effect of albumin in the body, sulfadiazine and sulfathiazole are the most effective sulfonamides for the treatment of meningitis as well as other infections.

Penicillin K is approximately 95 per cent bound in plasma, and penicillins G, F, and X approximately 50 per cent (Tompsett et al., 1947). Penicillin K, however, does not have significantly greater antibacterial activity than the less highly bound penicillins, in contrast to the situation encountered with the sulfonamides. Penicillin K is therefore less active in the body per unit concentration. The penicillins and streptomycin are not freely diffusible into the CSF, as are the sulfonamides, but produce much lower concentrations in the CSF than can be accounted for by plasma binding. They must consequently be administered intrathecally in treating meningitis. It is partly because of the ready penetration of the unbound fraction of the sulfonamides into the central nervous system that these drugs remain of great value in treating meningitis, even though penicillin has largely supplanted them in treating other types of infection.

The physiologic significance of binding by plasma proteins has been discussed in greater detail elsewhere (Davis, 1948). For other aspects of the absorption, distribution, and excretion of chemotherapeutics, a text on pharmacology should be consulted.

#### INHIBITORS IN TISSUES

As has been noted, sulfonamides are antagonized by extremely low concentrations of p-amino benzoic acid, and by larger concentrations of purines and other compounds; they are bound by serum albumin and probably other macromolecules. The concentration of these substances in the tissues in most infections is not great enough to interfere with chemotherapeutic activity. In the presence of pus, however, sulfonamides are quite worthless; the nature of the

substances responsible has not been determined. In uninfected wounds, products of tissue autolysis appear to play a similar, though perhaps less decisive, role. At any rate, though sulfanilamide, applied locally and systemically, was routinely used in many thousands of wounds in World War II, the Committee on Wound Infection of the National Research Council finally concluded that its prophylactic action against wound infection had been questionable. Fortunately, penicillin is relatively unaffected by pus or products of tissue breakdown. For best results, however, the surgical drainage of pus is still considered mandatory, although there is some indication that local infiltration of penicillin may even eliminate the necessity of incising certain types of purulent infection. The low pH of pus (6.2 to 6.5) and wounds may be an important factor in tissue inhibition of the antibacterial action of sulfonamides and streptomycin. Sulfonamides show a tremendous loss of activity at pH 6.0. Penicillin, however, an acidic compound, is even more active at acid pH.

#### LOCAL VERSUS SYSTEMIC THERAPY

The chemotherapeutics penetrate quite rapidly into all the tissues of the body which are adequately bathed by transudate from the circulation. Nevertheless, when infected surfaces can be treated locally, it is often preferable to obtain higher concentrations at the site of infection by using the drugs as surface antiseptics, with or without systemic chemotherapy. The clinical details will not be discussed here, but the following examples may be mentioned: superficial skin infections (e.g., sulfathiazole or penicillin for impetigo); conjunctivitis; mouth and throat (e.g., penicillin mouth wash or lozenges for superficial cases of Vincent's angina; and penicillin or tyrothricin spray for diphtheria carriers); tyrothricin for varicose and other chronic skin ulcers. It must be emphasized, however,



that local treatment should not be substituted where systemic treatment is required; this abuse of the convenience of local treatment is a great temptation in home and office practice.

In addition, high local concentrations can be induced with advantage in several internal locations. Chronic pulmonary infections are frequently improved by nebulized penicillin sprays (aerosols); this treatment, which can be self-administered by the patient, promises to be extensively developed. "Intestinal antiseptics" are useful in the treatment of certain enteric infections and in partial sterilization of the gut prior to gastro-intestinal surgery. These include streptomycin given orally, and certain sulfonamides (e.g., succinyl sulfathiazole) which are poorly absorbed and hence maintain high intestinal concentrations without toxic effects from excessive absorption. There may be some advantage in infiltration of penicillin in tissues surrounding a localized infection. Finally, the concentration of the chemotherapeutics excreted in the urine is generally considerably greater than that in the body fluids. Consequently the chemotherapeutics are effective in smaller doses than usual when used as urinary antiseptics for the treatment of cystitis or superficial pyelitis.

#### CO-OPERATION WITH NATURAL DEFENSES; CAUSES OF FAILURE OF CHEMOTHERAPY

It is hardly a coincidence that the greatest successes of chemotherapy have been attained in acute diseases, in which natural defenses, if brought into play in time, are by themselves able to bring about a rapid and permanent cure. The most conspicuous failures of chemotherapy are chronic infections: tuberculosis, the late stages of syphilis (except neurosyphilis), brucellosis, systemic mycoses, relapsing vivax malaria and other chronic protozoal infections. A fairly high proportion of cures has been achieved by penicillin against two diseases whose

chronicity appears to be due chiefly to their location rather than the characteristics of the organism: osteomyelitis and subacute bacterial endocarditis.

The mechanism of chronic infection, in which neither the host nor the parasite gains a decisive victory, is one of the major problems of infectious disease, and cannot be discussed in detail here. Possible mechanisms include failure or slowness of the host to develop an effective immune response; protection of the parasites from circulating defense mechanisms, humoral and cellular, by intracellular location or by avascular pathological tissue; and development by the parasite of special resistant forms which lie dormant in the tissues (e.g., the cryptozoites of malaria, which have no known parallel among those bacteria which do not form spores). The parallel resistance of certain diseases to natural defense mechanisms and to chemotherapeutic cure suggests that the latter may depend upon the former. Indeed, it has been shown experimentally that phagocytosis plays a distinct role in the cure of pneumococcus pneumonia of mice by sulfonamides. As these drugs are primarily bacteriostatic in their action in vitro, it is not surprising that immune mechanisms should be required for elimination of the parasites in the host.

The action of some other chemotherapeutics, however, involves a bactericidal effect, both in vitro and in vivo. It is not unusual in penicillin-treated patients, for example, to demonstrate pneumococci on the smear of a pleural fluid which is sterile on culture, the bacteria having been sterilized without benefit of phagocytosis and probably without benefit of antibodies. Yet clinically, with the bactericidal antibiotics as well as the bacteriostatic sulfonamides, it is often necessary to continue chemotherapy for quite some time beyond the initial overwhelming attack on the bacteria. Moreover, chemotherapeutic failures are common with some acute infections caused by susceptible organisms, such as *Klebsiella pneumoniae* and various types of meningitis.

Why do bactericidal drugs fail to eliminate some infections rapidly? Two mechanisms may be suggested. The parasites may be protected by their location from chemotherapy as well as from immune mechanisms. This mechanism is particularly plausible for intracellular organisms and for severe acute infections, where the rate of penetration may be the decisive factor (e.g., purulent meningitis). Sec-

ondly, since penicillin is bactericidal exclusively to dividing organisms, and streptomycin chiefly to actively metabolizing ones, any organisms which might be located at nutritively inadequate sites would be resistant to sterilization. Support for this explanation is offered by the observation in vitro of a tiny fraction of "persisters," organisms which fail to be sterilized after even 24 or 48 hours of exposure to penicillin or streptomycin. As their progeny are not particularly drug-resistant, this phenomenon appears to depend on the physiologic or nutritive state of these surviving cells, rather than on the genetic development of drug resistance.

Unfortunately, there is little real knowledge on this question. One fact is clear: it is easier to produce an immediate chemotherapeutic response than to eradicate an infection. In some cases this phenomenon involves development of drug-resistant organisms. In most cases we must search elsewhere for the cause and for methods to circumvent it.

The "chemotherapeutic paradox" (inactivity in vitro of drugs known to be effective in vivo) was very disturbing to earlier workers (Dale, 1923), and led to considerable speculation concerning the possible role of host mechanisms. Conversion in the body of inactive arsenicals to active trivalent arsenoxide derivatives was early shown; but similar mechanisms were not demonstrated for germanin and various dyes which were active against trypanosomes only in vivo. More recently penicillin, which is known to act directly on bacteria, could not be shown to affect the treponemes of syphilis in vitro. It seems likely that the inability to provide the conditions necessary for the multiplication of these parasites may account for the failure to demonstrate chemotherapeutic action against them in vitro. In any case, the weight of modern evidence has amply demonstrated that Ehrlich was correct in the view, unpopular for several decades, that chemotherapeutics, in original or altered form, act directly on the parasites. This view, of course, by no means overlooks the co-operation of independent host mechanisms in eliminating inhibited parasites.

A few words might be in order here to recapitulate the reasons for the complete chemotherapeutic failure of many compounds, natural and synthetic, which are active in vitro in high dilution. In a sense it is true that toxicity is always the limiting factor. This statement is true by definition since the dosage is always tested up to the level of toxicity, and chemotherapeutic failure is the failure to exert antimicrobial action at doses below the toxic level. But if one inquires more closely into such failures, it becomes apparent that in many instances, such as the dyes and surface-active agents, the reason for failure is not the extraordinary toxicity of the compounds, but rather their inactivity at doses which would be expected to be active were the compounds freely distributed and active in the body fluids. Binding to plasma proteins neutralizes their activity; binding to tissue constituents neutralizes them and, in addition, may or may not be toxic, depending on the site of the interaction. In addition, metabolic antagonists produced by the host may also render some compounds ineffective (e.g., sulfonamides in pus). This has been suggested as the reason for the chemotherapeutic failure of the many competitive metabolite analogues which have been synthesized and found to be active only in vitro.

## DRUG RESISTANCE

### OCCURRENCE

Ehrlich encountered this phenomenon with trypanosomes and studied many aspects which have been rediscovered with antibacterial agents. Strains of parasites recovered from inadequately treated mice were found to display varying degrees of resistance to the drug; resistance to each of the three chemical series studied (fuchsin dyes, azo dyes such as trypan red, or arsenicals) could be produced separately or together in a single strain; resistance ex-



tended to all the members of a given series, implying affinity for the same receptors. Resistant strains showed a decreased fixation of the drug (a phenomenon which has not been demonstrated for modern antibacterial chemotherapeutics).

It should consequently not have been surprising when sulfonamide-resistant ("drug-fast") strains of previously susceptible bacterial species appeared after the introduction of sulfanilamide, and later also in greater or less degree with the other chemotherapeutics. Resistance may be produced quite regularly in vitro with most bacterial species by successively transferring organisms to media containing increasing concentrations of drug. At varying stages in this process strains are obtained with varying degrees of permanent resistance. In some cases (e.g., *H. influenzae* and streptomycin), in addition to this gradual development of a 2-fold to a 4-fold increase in bacteriostatic concentration at each transfer, a much more dramatic change occurs, with the production in a single step of colonies permanently resistant to a thousandfold increase in concentration. Resistant strains may similarly appear gradually or suddenly in treated patients or animals whose infection has not been rapidly eradicated. Exposure to the drug without bacterial multiplication, in vitro and presumably in vivo, does not lead to development of resistance. The development of resistance has not been observed to any appreciable extent with disinfectants (other than dyes) or heat; increased resistance to radiation has been reported recently.

The phenomenon of drug resistance presents two major theoretical problems: (1) how does the resistant cell differ physiologically from its sensitive parent; (2) by what mechanism does this difference originate?

#### PHYSIOLOGIC MECHANISM OF RESISTANCE

A few sulfonamide-resistant strains have been shown by Landy to synthesize an in-

creased amount of PABA, and a few penicillin-resistant strains produce an enzyme (penicillinase) which destroys the drug. An interesting observation by Kohn and Harris is the loss of the capacity to synthesize methionine by certain *E. coli* strains, not originally requiring methionine, which have become sulfonamide resistant in a medium containing methionine. Sulfonamides apparently inhibit methionine synthesis; growth in the presence of this nutrilit and drug permits survival of bacterial variants which have lost this particular drug-sensitive metabolic mechanism. In the vast majority of bacterial strains investigated, however, no changes have been observed which are so obviously related to drug resistance; the detection and analysis of whatever changes do exist could be expected to throw much light on the mode of action of the drugs. In a few cases metabolic or morphologic changes (e.g., alteration of capacity to ferment sugars) have been observed. In particular, staphylococci very resistant to penicillin (6,000 units/ml.) were found to have lost the capacity to accumulate free amino acids intracellularly (Gale and Rodwell, 1948), to grow anaerobically, to utilize a variety of carbohydrates, and to stain Gram positive. Some resistant strains of various organisms grow more slowly or are less virulent than their parent strain. But none of these changes have been very directly related to the action of the drug.

We are therefore limited in most cases to the very general assumption that an altered metabolic pattern has been produced in the cell. This could involve an alteration in the concentration of the various members of a group of enzymes which successively deal with a series of steps in a synthetic process—a quantitative alteration, stressed by Hinshelwood (1946), which would cause increased accumulation of some metabolite antagonistic to the drug. This could account for the wide range of degrees of resistance observed. A more qualitative possibility, not sharply demarcated from the other, is the substitution of an alternate metabolic pathway toward a certain product. This notion is most attractive

in those cases in which a dramatic change in resistance has suddenly developed. An example which could fit either hypothesis is McIlwain's observation that the diphtheria bacillus, which requires pantothenic acid, is inhibited by its sulfonic analogue, pantoyl taurine, while a variant which no longer requires pantothenic acid, having developed the capacity to synthesize it, was found to be insensitive to pantoyl taurine. But one cannot generalize from this observation, as most sulfonamide sensitive bacteria synthesize enough PABA to satisfy their own normal requirements.

It has also been suggested that resistance may depend on reduced permeability to the drug, which would involve a structural as well as a metabolic alteration. This interpretation is not very convincing, since a partial decrease in permeability would slow the rate of penetration but would not affect the concentration producing bacteriostasis at equilibrium; while a complete elimination of permeability would not account for the varying degrees of resistance observed. We agree with Ehrlich's emphasis on change in intracellular structure rather than a change in permeability, but for his vague concept of decreased avidity of the receptors the modern concept of alteration in the concentration of enzymes is substituted.

#### ORIGIN OF RESISTANCE

The mechanism of production of the resistant strain is generally assumed to be natural selection (i.e., differential multiplication) of a resistant variant spontaneously arising in the course of bacterial multiplication, the drug acting merely as a selective inhibitor of the cells which have failed to develop the appropriate mutation. Similar phenomena of spontaneous bacterial variation in fermentative capacity, antigenic structure, etc., are discussed elsewhere in this book (pp. 27-30). The number of individual cells in bacterial cultures is so enormous that the frequency of mutation required to explain the development of drug resistance is very low, certainly no greater than the frequency of spontaneous mutation observed in the germ cells of higher plants and animals. There is little question that this genetic explanation applies to the

instances of dramatic change in drug resistance. For example, Alexander and Leidy (1947) have shown that in a culture of *H. influenzae* sensitive to 1  $\mu\text{g.}/\text{ml.}$  of streptomycin, approximately 1 out of  $10^{10}$  cells are resistant enough to produce a colony on a plate containing 1,000  $\mu\text{g.}/\text{ml.}$  Similar phenomena have been shown with shigella and other organisms. The more gradual and limited adaptation of *S. aureus* to penicillin has also been analyzed along genetic lines, indicating that the process of gradual adaptation also involves a whole series of discrete mutations (Demerec, 1945). Hinshelwood (1946), however, has suggested that the gradual production of increased resistance is not based simply on spontaneous mutation, but involves an inheritable physiologic alteration of the cellular enzyme pattern in which the drug plays a directing as well as a selecting role.

Although the Lamarckian concept of inheritance of acquired characteristics has long been eliminated with respect to the gross morphologic characteristics studied by earlier biologists, we cannot deny a priori its possibility when the acquired characteristic may be induced chemically in a germ cell—in this case, a bacterium. In the usual classification of bacterial adaptations, e.g., formation of adaptive enzymes, a rigid distinction is drawn between the process of what might be called genetic adaptation, which produces a permanent, inheritable effect based on spontaneous mutation, and the process of physiologic adaptation, in which the bacterial enzyme pattern reverts to the original state as soon as the stimulating substrate is withdrawn. Hinshelwood's observations suggest that this classification may be somewhat Procrustean. It must be emphasized, however, that the concept of a directed, yet inheritable, physiologic adaptation is quite controversial. While this concept conflicts with the principles of classical genetics, which are being applied with striking success to many phenomena of bacterial variation, it does have some precedent in the phenomena of morphogenesis (embryologic differentiation). Here the environments of the various parts of the unfolding organism somehow guide the nature of the differentiation, and yet these "adaptive" cellular changes are stable and essentially irreversible.



## PRACTICAL IMPORTANCE

The rate of development of drug resistance varies widely with bacterial species and chemotherapeutic agent. No general rule is possible beyond the statement that streptomycin is much the worst offender, sulfonamides next, and penicillin remarkably free of this as of other objections. *S. aureus* is particularly prone to develop resistance to penicillin, while hemolytic streptococci are practically incapable of any such alteration. The phenomenon is of importance in two ways, individual and epidemiologic.

In the treatment of the individual case the development of a high degree of resistance naturally destroys the effectiveness of therapy. It is believed that this development may be minimized by treating with adequate doses to provide a margin of safety in the concentration in the body, which will not permit therapeutic "escape" following development of slight resistance. Such escape is rarely a problem in septicemic diseases or pneumonia. On the other hand, in a situation where highly resistant organisms appear quite regularly, as in *H. influenzae* meningitis treated with streptomycin, it is a matter of chance whether the organisms already present include resistant cells; streptomycin may consequently either cure rapidly or permit relapse, regardless of dosage. Development of drug resistance is apt to be a particularly important problem in the prolonged treatment of a chronic disease, such as tuberculosis, where a preponderance of resistant organisms appears fairly regularly following 1 to 3 months of treatment with streptomycin. This may actually represent quite rapid development of resistance, in terms of the number of bacterial generations involved. The presence of resistant tubercle bacilli in the sputum, however, does not exclude the existence of foci of susceptible organisms as well.

Mention has already been made of the report that some resistant tubercle bacilli,

isolated from patients, appear to be stimulated in their growth in vitro by streptomycin, while some resistant strains of meningococci and other organisms actually require this compound. The continuation of therapy with this drug after the development of a large number of resistant organisms is therefore not only useless, but in some cases a positive hazard.

The epidemiologic problem concerns the possibility of widespread distribution of resistant organisms as a result of a process of selection, following alteration of the parasite's ecology by the widespread use of the drug among its hosts. This process limits the value of sulfonamide prophylaxis of respiratory infections or carriers, such as  $\beta$ -hemolytic streptococcus and meningococcus, in crowded populations. The most significant example of this phenomenon at present is the result of sulfonamide treatment of gonorrhea. This disease presents a particularly favorable situation for such a development, since the absence of an animal reservoir or of frequent human carriers eliminates these sources of organisms unexposed to the drug, while the absence of immunity permits repeated reinfection. In the New York City Health Department Clinics from 1936 to 1942 the proportion of cures achieved with sulfonamides dropped steadily from approximately 90 per cent to 30 per cent. Had penicillin not been developed, the chemotherapy of this disease would have become practically useless. Since most resistant bacteria appear to be as virulent as their sensitive parents, and show little tendency to lose their resistance in vitro or in vivo, resistant strains persist in the population.

On the basis of evolutionary theory it can be predicted that among those bacterial species which are drug-sensitive as encountered in the "wild" state in nature, the drug-resistant variants have relatively less survival value—survival depending on virulence, growth rate, nutritive requirements, etc. Otherwise the species would have been drug-resistant in the first place (as are many other species), since

variants of decreased and increased resistance may safely be assumed to have appeared continually through the millennia. But the quantitative differences in survival value may be too slight to have much selective effect on the population over the relatively short periods of time, measured in years or decades, which interest epidemiologists. It is of interest in this connection to note that a particularly wide range of susceptibility to a drug is observed in nature (previous to therapy) with organisms (e.g., staphylococcus and penicillin) from which drug-resistant, virulent strains readily emerge under the influence of the drug. In such a case, it may be concluded that moderate drug resistance can be associated with no loss of survival value. But since two strains with identical degrees of resistance are not necessarily metabolically identical, it does not follow that all equally resistant strains of such a species have equal virulence or survival value.

#### COMBINED THERAPY

The development of resistance to any one of the various drugs (considering the sulfonamides as a single group) is independent of resistance to the others. While it is possible to develop resistance to one drug in organisms already resistant to another, it is practically impossible to develop in the presence of both drugs a strain resistant to either—presumably because of the infinitesimal chance of simultaneously developing both mutations in a single cell. In consequence, there is excellent theoretical justification for combined therapy where two effective drugs are available and development of resistance is an appreciable danger. Combined therapy also has probable advantage where the drugs differ in their distribution in the infected tissues—e.g., penicillin plus sulfadiazine in treating meningococcus meningitis. The traditional view, that the simplest therapy is necessarily the best, has served a useful purpose in purging mystic concoctions from the pharmacopeia, but must not be arbitrarily applied to the problem of obtaining the best clinical results from a variety of available effective chemotherapeutics.

## CHEMOTHERAPEUTIC AGENTS IN USE

### SULFONAMIDES AND SULFONES

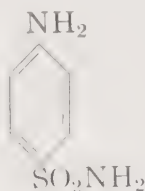
Since p-amino benzene sulfonamide (sulfanilamide) was established as the effective portion of the Prontosil molecule, a large number of active derivatives have been synthesized by substitution of the amide group. While sulfanilamide was limited in its effectiveness practically to streptococcus, *E. coli*, and neisserial infections, the more powerful derivatives were found to be active against a very wide variety of pathogenic organisms. All the sulfonamides appear to have the same mode of action and are antagonized by p-amino benzoic acid; there is no specific relation between any member of the group and any particular organism. Sulfanilamide was therefore largely superseded in turn by the more active sulfapyridine and sulfathiazole. While sulfathiazole is the most active sulfonamide known per unit concentration, it is less widely used today than the less toxic and almost equally active sulfadiazine or the monomethyl and dimethyl derivatives of the latter, sulfamerazine and sulfamethazine. The pharmacologic problem of toxic reactions will not be discussed here.

The organisms against which sulfadiazine and similar compounds are usually effective, in concentrations possible in the body, include:  $\beta$ -hemolytic streptococcus, pneumococcus, meningococcus and gonococcus, staphylococcus, the coli group, shigella and cholera vibrio, anthrax bacillus, diphtheria bacillus, and the hemophilus group; also some cases of actinomycosis and of infection with the large viruses of trachoma and lymphogranuloma inguinale. The effectiveness against staphylococci is limited, especially since sulfonamides are inactive in the presence of pus. The drugs are also active in vitro, but rarely of clinical value, against  $\alpha$ -hemolytic streptococci, clostridia, salmonella, brucella, and the tularemia, proteus, and pyocyaneus organisms. They are



inactive, except in prohibitive concentrations, against anaerobic Streptococci, spirochetes, mycobacteria, rickettsiae, and most fungi and viruses. They are active in *Plasmodium knowlesi* malaria of monkeys, but are relatively ineffective in human malaria because the large doses required produce excessive concentrations in the urine in tropical climates.

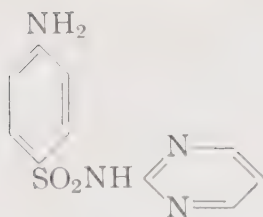
penicillin in the treatment of such common diseases as streptococcus or staphylococcus infection, pneumococcus pneumonia, and gonorrhea. It is unfortunate that the ease of oral administration of sulfonamides leads many practitioners to prescribe them in upper respiratory infections. There is no evidence that the action of sulfonamides on secondary bacterial invaders warrants the



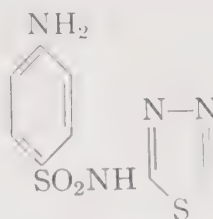
Sulfanilamide



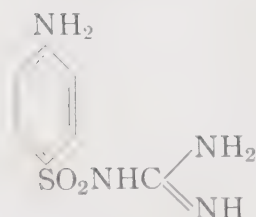
p-Amino benzoic acid



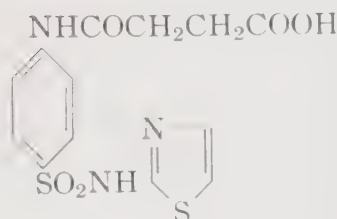
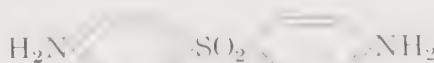
Sulfadiazine



Sulfathiadiazole



Sulfaguanidine

Sulfasuxidine  
(succinyl sulfathiazole)

Diamino diphenyl sulfone

The usual clinical dosage in adults is of the order of magnitude of 1 Gm. per 4 hours, following a larger initial saturation dose; this regimen produces an average blood level of 5 to 10 mg. per cent. While sulfonamides affect a larger variety of organisms in vitro than penicillin, in practice most of the clinical infections susceptible to sulfonamide treatment are also responsive to penicillin, with less toxicity and more rapid and certain effect. For this reason sulfonamides are being increasingly replaced by

use of these fairly toxic drugs in such self-limiting virus infections.

Sulfonamides appear at present to be the drug of choice in the treatment of the following conditions:

**Meningococcus meningitis.** Not only is this organism at least as susceptible to sulfadiazine as to penicillin, but the sulfonamide penetrates more freely into the cerebrospinal fluid.

**Certain systemic and urinary tract infections with *E. coli*.**

**Bacillary dysentery, cholera, and intestinal antiseptics** in preparation for gastro-intestinal surgery. For these purposes sulfadiazine may be used, or large doses (2 to 3 Gm. per 4 hours) of sulfonamides which are poorly absorbed or which slowly release sulfathiazole. This group of intestinal antiseptics includes sulfaguanidine, sulfasuxidine (succinyl sulfathiazole), sulfathalidine (phthalyl sulfathiazole) and sulfathiadiazole. It may be noted that competition for this purpose is now offered by oral streptomycin, which appears to accomplish the same inhibition of the predominantly Gram-negative flora of the intestine in a shorter period of time.

**Chemoprophylaxis.** Sulfadiazine or similar compounds in low dosage (ca. 1 Gm. per day) may be given for periods of years with little toxicity, and appears to be of value in preventing recurrences of rheumatic fever associated with infection by  $\beta$ -hemolytic streptococci. A single dose of sulfadiazine has been reported to clear up 90 per cent of meningococcus carriers.

Diamino diphenyl sulfone, a derivative in which the amide group has been completely eliminated, proved too toxic for clinical use. Its thiazole derivative, promizole, and the di-glucose sulfonate derivative, promine, are reported to have some value in treating leprosy. Their beneficial effect on experimental tuberculosis was not paralleled clinically since the low therapeutic index did not permit high enough dosage.

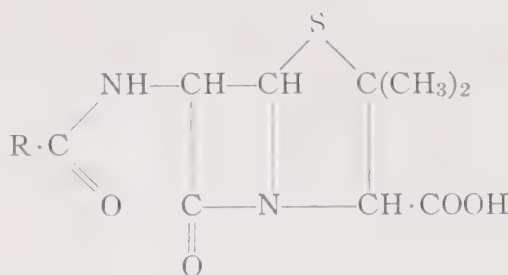
A curious outgrowth of sulfonamide research was the discovery that p-amino benzoic acid (PABA) in large doses (3 Gm. every 4 hours) exerts some chemotherapeutic activity against rickettsial infections. This interchange of the role of drug and antagonist is certainly evidence of the intimate metabolic role of these compounds. A closely related compound, p-amino salicylic acid (PABA containing a phenolic OH group) markedly stimulates the respiration and inhibits the growth of tubercle

bacilli, and has shown a beneficial effect on experimental tuberculosis. It is antagonized by PABA.

### PENICILLIN

The accidental discovery of this remarkable substance by Fleming in 1929, and its subsequent purification and testing in 1940 by Chain and Florey, have already been mentioned. The initial yield was very low. The circumstance of its development during the course of World War II led to organized governmental subsidy of its practical production; as a result the American fermentation industries were able to produce huge amounts at relatively low cost within a matter of one to two years.

Following are the structures of the known penicillin molecules:



<i>R group</i>	<i>Penicillin</i>
Benzyl. ....	G
p-Hydroxy benzyl. ....	X
n-Pentenyl (5 C).....	F
n-Heptyl (7 C).....	K

It will be noted that the ring structure consists of two amino acids, alanine and  $\beta$ -dimethyl cysteine (thiovaline). These were found on isolation to be in the d configuration "unnatural" to animal tissues. (The rings can alternatively be pictured as arising from condensation of cysteine and valine.) The penicillins are fairly strong acids, of pK approximately 2.8, and are distributed commercially as the sodium or calcium salt. These are stable in the dry state but deteriorate within a few days in solution even in the refrigerator.

The drug was originally produced from surface cultures of the mold *Penicillium notatum*, a technic which does not lend itself to large-



scale industrial work. Better results are now obtained with vigorously aerated deep vat cultures. It has been found possible to increase the yield by selection of appropriate strains, including the use of mutants induced by ultraviolet irradiation. The organisms in use at present produce over a hundred times the yield of Fleming's original strain. It is also possible to favor the production of the desired compound (at present penicillin G) by appropriate composition of the culture medium.

The original Oxford unit was defined as the amount required in 50 ml. of meat extract broth to inhibit the growth of a standard strain of *Staphylococcus aureus* H. This is equivalent to 0.6  $\mu$ g. of crystalline penicillin G. The standardization of the various compounds has been somewhat confused; not only do they vary in their binding to serum albumin and hence their activity in the body (page 659), but penicillin X, which is only approximately one-half as active per unit weight as penicillin G against the standard organism, is somewhat more active against some other organisms. This slight relative specificity of the various penicillins, however, though important for accurate standardization, does not appear to be clinically significant.

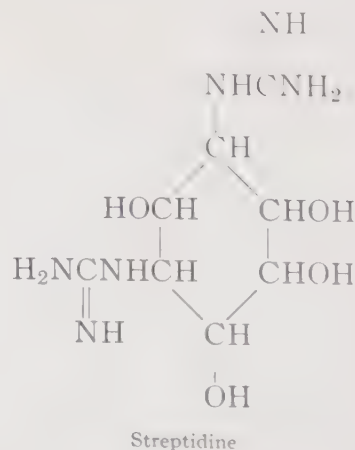
Penicillin is active against practically all Gram-positive organisms, including the anaerobic and  $\alpha$ -hemolytic streptococci, which responded poorly to sulfonamides in subacute bacterial endocarditis and chronic pulmonary suppuration. In addition, it is effective against the Gram-negative gonococcus and meningococcus, various spirochetes (syphilis, Vincent's angina, relapsing fever and leptospirosis) and actinomycetes. It has had a striking effect on the technic of surgery in nonsterile areas (e.g., pulmonary surgery), and on the results obtained in war wounds and surgical infections, since the most important pathogens in these mixed infections are usually staphylococci and streptococci. Penicillin, unlike the sulfonamides, is effective against these organisms even in the presence of pus, but chemotherapy is not a substitute for surgical drainage of localized pus. In diphtheria penicillin shortens convalescence and inhibits development of the carrier state but does not substitute for antitoxin.

The dosage varies widely, but it is usually between 100,000 and 400,000 units per day, intramuscularly in divided doses. Much larger doses are well tolerated but are usually unnecessary. Penicillin can be given orally, but with considerable waste (the required dosage being about 5 times as large) and with irregular absorption. In the treatment of meningitis it must be given intrathecally. Although serious toxic effects of penicillin are rare, allergic skin reactions are quite frequent. It is just as inexcusable to prescribe penicillin as sulfonamides in ordinary respiratory infections.

The development of drug-resistant strains in the course of treatment with penicillin has so far been a negligible danger, with the possible exception of staphylococcus. Species of *S. aureus* encountered in nature vary in their inhibitory requirement from 0.02 units per ml. to several hundred times that value. Other susceptible species vary in their requirement from 0.01 to 10 units ml.

#### STREPTOMYCIN

This chemotherapeutic, produced by *Streptomyces griseus*, was discovered in 1944 by Schatz, Bugie and Waksman in the course of a systematic search for antibiotics from molds. The streptomycin base yields on hydrolysis the following products:



Streptidine

+ Streptobiosamine (disaccharide of methyl glucosamine + streptose)

As the two guanidine groups substituted on the inositol ring of streptidine are strong bases, streptomycin is distributed commercially as the hydrochloride, sulfate or calcium chloride complex. It is quite stable in solution but cannot be autoclaved without loss of potency. The original S unit was found to equal 1  $\mu$ g. of the pure base.

The presence of streptomycin in the therapeutic armamentarium is very nicely complementary to penicillin, since it is effective against most of the important Gram-negative pathogens, including *H. influenzae* (meningitis, endocarditis, respiratory infection), tularemia, plague, Friedlaender's (*Klebsiella*) pneumonia, urinary tract infections with colon, proteus and pyocyanus organisms, and shigella dysentery. It is also effective but not regularly curative against the Gram-negative organisms of mixed pulmonary infections, liver abscesses, bile-duct infections and peritonitis. *Salmonella* meningitis responds, but the results in ordinary typhoid or paratyphoid fever, as also in brucellosis, have been disappointing. Spink has reported that streptomycin together with a sulfonamide is much more effective in brucellosis than either drug alone.

In tuberculosis promising results have been obtained in experimental animals, and dramatic relief in miliary tuberculosis and tuberculous meningitis in man, which are ordinarily rapidly fatal. Primarily exudative pulmonary lesions, tracheo-bronchial ulcers, and tuberculous draining sinus tracts frequently respond, at least temporarily; the drug is much less effective, however, against the fibro-caseous lesions typical of human chronic pulmonary tuberculosis.

Susceptible species vary in their antibacterial requirement from 0.01 to 10  $\mu$ g. per ml.

The usual dosage of streptomycin is about 2 Gm. per day, intramuscularly in divided doses, or orally for enteric infections or surgical prophylaxis. In treating meningitis it is given intrathecally as well as systemically. Somewhat smaller doses are used in the pro-

longed treatment of tuberculosis. Streptomycin is not nearly so ideal a drug as penicillin: resistant strains of bacteria are found widely in nature and develop rapidly in the course of treatment, and the drug is quite toxic. In addition to minor symptoms, after treatment for several weeks vestibular dysfunction appears quite regularly, and is sometimes irreversible; deafness is occasionally also produced.

These toxic effects, as well as the fairly regular appearance of drug-resistant tubercle bacilli following 1 to 3 months of treatment, limit the period of effective therapy of tuberculosis; the beneficial effects in this chronic disease are therefore often quite transient. The role of streptomycin in the treatment of tuberculosis is under extensive investigation; the consensus of present opinion is that its toxicity does not justify its use in minimal cases, but it may be of value in more advanced cases.

The pharmacology and clinical use of streptomycin have been reviewed by Murray et al. (1947) and by Paine et al. (1947).

#### TYROTHRIN: GRAMICIDIN AND TYROCIDINE

Gramicidin and tyrocidine were crystallized by Hotchkiss and Dubos from a mixture called "tyrothrin," obtained by Dubos in 1939 from strains of a Gram-positive sporulating soil bacillus (*B. brevis*) and trained to grow on a medium enriched with Gram-positive organisms. They are both polypeptides with a molecular weight of 1,500 to 2,500, and both contain d-amino acids. Tyrocidine has free basic and acidic groups and consequently is somewhat soluble in aqueous solution; it is effective in high dilution against both Gram-positive and Gram-negative organisms. It behaves in several respects, including its rapid bactericidal effect and its lytic action on organisms, like a cationic detergent. Gramicidin exerts a more selective metabolic effect. Its bacteriostatic and bactericidal activities are restricted to Gram-positive or-



ganisms, although meningococci and gonococci are also extremely susceptible to it. It has no ionizing groups and is quite insoluble in water. Contrary to what is observed with ordinary antiseptics, its antibacterial activity is markedly increased in the presence of serum albumin, at both acid and alkaline reactions, and therefore persists in the presence of body fluids and tissues. The toxicity of tyrothricin and gramicidin, especially their hemolytic effect, has restricted their use in man to local antiseptics (for example, the treatment of chronic ulcers) and in animals to the local treatment of bovine mastitis. Nevertheless they hold an honored historic position as the first antibiotics of some practical value. Their chemistry and antibacterial action have been reviewed by Hotchkiss (1944). A similar compound, gramicidin S (S standing for Soviet), has been isolated by Gause and Brazhnikova.

#### OTHER ANTIBIOTICS

**Lysozyme** is a protein widely distributed in nature, discovered by Fleming in 1922, which occurs in especially high concentration in egg white and tears. It causes rapid lysis of susceptible organisms by enzymatic hydrolysis of a mucopolysaccharide, but has not been of chemotherapeutic value since the highly susceptible organisms are not pathogenic. Indeed, it may well be the presence of lysozyme in the body which robs these organisms of pathogenicity. Animals undoubtedly produce other similar antibacterial agents, in addition to antibodies and complement, which are well worth searching for.

**Bacitracin** is an antibiotic obtained by Johnson and co-workers in 1945 from a strain of *B. subtilis* contaminating a patient's wound; its recognition was based on the interesting observation that certain pathogenic species appeared in plate cultures but not in broth cultures taken from the wound. It has much the same antibacterial spectrum as penicillin and is reported to have little toxicity.

**Subtilin**, obtained from another *subtilis* strain by Salle in 1945, is reported to have similar properties, and may be the same compound.

**Polymyxin and aerosporin** are two very similar polypeptide products of bacteria which are reported to be quite nontoxic and effective against various Gram-negative organisms. Preliminary clinical results indicate effective action against pertussis.

**Chloromycetin** is a mold product reported to be especially effective against rickettsiae, as well as many bacteria.

**Aureomycin**, another mold product, is also effective against rickettsiae and the large viruses which approach the rickettsiae in size (the trachoma-lymphogranuloma group).

It is still too early to assess the place in chemotherapy of these antibiotics, which at the time of this writing are not yet commercially available; further promising new ones are likely to continue to appear. Not only are new diseases being brought under control, but some of these substances may turn out to be the drug of choice in the treatment of certain infections which already respond quite well to penicillin or streptomycin. This situation raises a difficult problem in clinical research. As was emphasized earlier in this chapter, the evaluation of a chemotherapeutic, after satisfactory performance in infected animals, must ultimately depend on clinical testing—and one hesitates, in testing a new drug against a life-threatening disease, to withhold a well-known agent of definite though imperfect value. But even where these new chemotherapeutics fail to supplant the older ones, it will be useful to have additional powerful agents against various bacteria; this will permit substitution in the treatment of resistant organisms, or combined therapy to prevent their emergence.

Although new antibiotics are reported in almost every month's journals, a sophisticated medical audience has seen too many failures and now withholds enthusiasm until clear evidence of activity in vivo and low toxicity has been presented. The host of recorded toxic antibiotics will not be discussed here; some of these may be of value in selective cultivation or as metabolic reagents. The problems encountered in systematic search for antibiotics are discussed by Waksman (1947) the

chemistry of the known compounds by Oxford (1945) and the problems of antibiotic chemotherapy by Dubos (1944).

### OTHER SYNTHETIC CHEMICALS

The treatment of syphilis with arsenicals and bismuth, which are being largely replaced by penicillin, is discussed in the chapter dealing with this disease; arsenicals are still of great value in the treatment of trypanosomiasis. In this field it is of particular interest that the trivalent arsenoxide compounds, which were condemned by Ehrlich as being too toxic, have been re-investigated in recent years and proved to be superior to the trivalent reduced arsphenamines. Of the arsenoxides "Mapharsen" has been widely used, and even more promising derivatives recently developed by Eagle and collaborators. The arsenicals are active against many bacteria in vitro in high dilution but have not shown promise in antibacterial chemotherapy.

The principles involved in the chemotherapy of systemic parasitic infections are essentially the same as those encountered in antibacterial chemotherapy, except that in vitro testing is often impossible. While observations made in this field have been used in this chapter to illustrate theoretical points, the details will not be discussed here. Although present research in antibacterial chemotherapy emphasizes antibiotics, continuing success in the synthesis of increasingly effective antiprotozoal compounds offers encouragement for the hope that in antibacterial chemotherapy, too, sulfonamides will not long remain the only effective synthetic agents.

Table 53 summarizes the clinical responses of infections to the chemotherapeutics now commercially available. As experience increases and newer chemotherapeutics appear, the pattern will undoubtedly change.

TABLE 53. CHOICE OF CHEMOTHERAPEUTIC AGENT \*

INFECTIVE ORGANISM	SULFONAMIDE	PENICILLIN	STREPTOMYCIN
Gram-positive bacteria			
$\beta$ -hemolytic streptococcus.....	x	X	
$\alpha$ -hemolytic streptococcus.....	#	X	
Anaerobic streptococcus.....	0	X	
Staphylococcus.....	#	X	x
Pneumococcus.....	x	X	
B. anthracis.....	x	X	
Clostridia.....	0(?)	X	0
C. diphtheriae.....	x	X	
Gram-negative bacteria			
Meningococcus.....	X	x	
Gonococcus.....	x	X	x
E. coli.....	x	0	X
B. aerogenes.....	#	0	X
B. proteus.....	#	0	X
B. pyocyaneus.....	#	0	X
Salmonella.....	0	0	# (X in meningitis)
(including E. typhosa)			
Shigella.....	X	0	X
V. cholerae.....	X	0	0
Brucella.....	0(?)	0	#
P. pestis.....	X	0	x
P. tularensis.....	0(?)	0	X
H. influenzae.....	#	0(?)	X
H. pertussis.....	0	0	X
K. pneumoniae (Friedlaender).....	#	0	X
Ducrey's bacillus.....	X	0	
B. mallei.....	0	0	X
Acid-fast bacteria			
M. tuberculosis.....	#	0	X(?)
M. leprae.....	#	0	?
	(sulfone)		
Spirochetes			
Syphilis-yaws.....	0	X	
Leptospira.....	0	X	x
Borrelia.....	0	x	X
Spirillum minus.....	0	X	
Viruses, <i>except</i> .....	0	0	0
L. inguinale.....	X	0	
Trachoma.....	X	0	
Ornithosis-psittacosis.....	0	#(?)	
Granuloma inguinale.....	?	0	
Rickettsiae.....	0	0	0
Actinomycetes.....	x	x	0
Fungi and molds.....	0	0	0

X drug of choice.

x usually effective.

# effective under certain conditions.

0 ineffective.

Newer antibiotics not compared.

\* Adapted from table furnished by Dr. A. Goldstein, Department of Pharmacology, Harvard Medical School.



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## 36

# Principles of Epidemiology

### DEFINITION

By derivation, *epidemiology* would seem to be concerned with the explanation of epidemics of disease in human populations. While this definition still obtains, with the advance in biologic and medical science the field included under epidemiology has naturally broadened considerably (Frost, 1920; Maxcy, 1941). An epidemic is commonly defined as a sudden increase in the prevalence of a disease which is more or less constantly present or endemic in a community. To explain the sudden increase it is necessary to understand the factors which determine the usual or interepidemic levels of prevalence and the characteristic distributions which the disease ordinarily manifests in human populations.

While originally limited to infectious diseases, usage has extended the term to the study of diseases of unknown etiology, to diseases due to nutritional deficiencies, to senescence, to abnormal cell growth, and even to the casualties caused by physical and chemical agents, accidents, etc. In like manner, while originally limited to the phenomena of disease in human populations, usage has extended the term to the study of disease in animal populations and plant life. While they are essentially analogous, and may be related phenomena, there are cogent reasons why usage should sanction the distinction afforded by the employment of the more recently introduced terms "epi-

zootic" (epizootiology) for diseases of animals and "epiphytic" (epiphytiology) for diseases of plants.

Accordingly, it is proposed that the term epidemiology be reserved to designate the field of science dealing with the relationships of the various factors which determine the frequencies and distributions of an infectious process, a disease, or a physiologic state in a human community. For present purposes discussion will be limited to the principles of epidemiology as applied to understanding and control of human infections, particularly those due to bacteria.

### BIOLOGIC INTERPRETATION

The pandemic of influenza which occurred in 1918 was an appalling demonstration of man's helplessness and ignorance. It was obvious that epidemiology as a science was lacking in a valid rationale and a unifying concept. With the advances in the field of general biology and the collateral medical sciences, the needed concept gradually became evident. It was comprehensively formulated by Theobald Smith (1934) in the Vanuxem lectures delivered at Princeton.

Infectious disease is a manifestation of parasitism. Simple though this concept seems today, its formulation marked an important transition. The medical explanation of an infectious disease in man broadened to become a biologic one. It was real-



ized and accepted that it should not be set apart as peculiarly within the province of human medicine but should be viewed as an expression of the eternal struggle of living things for food by predation or parasitism, for shelter, and for propagation of their kind. More particularly, infectious disease is a reaction of one of the higher forms of life to the invasion of its tissues by some species of microparasite. This concept carries with it implications that are fundamental and far reaching. It affords a framework or pattern into which endless scattered observations can be fitted. Simply stated, it becomes apparent that the explanation of epidemic phenomena should be sought in the understanding of host-parasite relationships and the environmental factors which modify them.

### HOST-PARASITE RELATIONSHIPS

As a result of centuries of host wanderings, mutation and selective adaptation, the bacteria considered in this book have become established in the biologic orbit of man, and are responsible to a varying degree for some of his ills. Their potentialities range from those which are dependent upon certain human tissues and cells for their continuous propagation and survival, giving rise to common diseases with which mankind all over the world is familiar, to those which only inadvertently invade his tissues to cause an occasional sporadic case of a rare disease. The importance of each bacterial species to man has been determined by a few biologic principles to which only brief reference can be made.

Man is an obligate, a principal or an occasional host, according to the degree of success which a particular species of parasite has in passing through four critical stages in relationship to him. If a micro-organism has become completely dependent upon man for its survival, it is continuously successful (1) in finding entrance into his body through its proper portal of entry,

whether it be the mucous membrane of the respiratory, the alimentary or the genito-urinary tracts, or the skin by means of trauma or insect bite; (2) in reaching the particular organ, tissue or cells in which nutritive conditions are favorable for multiplication; (3) in making an exit from the body in excretions, secretions, or by blood-sucking insects; (4) in surviving under the conditions of the external environment, or in an insect vector, a sufficient time to reach a new susceptible host. To the extent that a microparasitic species is unsuccessful in continuously maintaining progressive passage through these four critical stages in human populations and their environment it must be able to utilize other host species or survival mechanisms. Thus, one of the first requisites of a rational explanation of the behavior of an infectious disease in a human community is to understand to what extent man as a host bears responsibility for the continuous propagation of the specific causative microparasite or shares this responsibility with other species animal, bird or insect.

### HOST REACTION

The second critical stage in the host-parasite relationship mentioned above is the one which may give rise to the symptoms and signs of illness by which the disease is recognized. The host reaction may vary greatly, both in severity and duration. A "case" of an infectious disease is a host reaction of sufficiently characteristic intensity and duration to permit clinical diagnosis. Reactions which are less intense and of shorter duration are called "abortive" or "suspected" cases, the clinical pattern being too indefinite or protean in nature to permit diagnosis, except in association with "frank" cases. When the subjective and objective symptoms are so slight as to pass unnoticed, the host is said to suffer from an "inapparent" infection. Infections which are below the threshold of clinical recognition are

grouped together as "subclinical." They can be identified only by laboratory procedures such as cultural recovery of the infecting micro-organism from the host's tissues, change in the reactivity of the skin to specific antigenic material, or change in a serologic reaction from a negative to a positive. It is at least theoretically possible that an infection may occur without demonstrable reaction on the part of the host, i.e., a symbiotic or a saprophytic relationship, but there is a difference of opinion as to whether the word infection should be used to describe such a condition.

### INFECTIOUS PERIOD

In clinical medicine, interest is centered upon the patient during the period that he is more or less incapacitated by the disturbance of physiologic functions caused by the invasion of a pathogenic micro-organism, that is, from the onset of symptoms to clinical recovery or to a fatal issue. In epidemiology, interest must be broadened to include the whole duration of the host-parasite relationship, that is, from the time of the infective exposure until the micro-parasite is suppressed or eliminated from the host's body. Of particular importance is the "infectious period," the time or times during which the microparasitic progeny are making an exit or are potentially available for transfer to a new host.

### CARRIERS

As early as 1890, Escherich noted that the infectious period of diphtheria was not necessarily coincident with the clinical course but that diphtheria bacilli might persist in the throats of patients during convalescence. In 1892, Guttman, Rommelaere and Simonds noted that cholera vibrios might be recovered from the feces during convalescence. Credit probably belongs to Koch (Winslow, 1943) for grasping the sig-

nificance of the fact that cases which could be clinically diagnosed were not alone responsible for the spread of contagious diseases. In his studies of cholera in Germany, during the winter of 1892 and 1893, he noted that some cases were so mild as to escape recognition, and indeed could only be detected with the aid of a bacteriologic investigation. The term "carrier" thus includes two classes. First, there are those who are about to have, or have already had, a clinical attack; they are designated as "incubatory," "convalescent" or "chronic" carriers. Second, there are those who are suffering from a subclinical or asymptomatic infection, the so-called "healthy" carriers. It is important to distinguish between these two classes, and for the purpose of this discussion, the second class of carriers will be included in the designation "subclinical" or "inapparent" infections.

### EPIDEMIOLOGIC PATHOGENICITY

From an epidemiologic viewpoint, the pathogenic potentialities of a given species of microparasite for the human host are roughly indicated by the proportion of clinical attacks which are fatal. Stated in different words, it is the ratio between cases and deaths (usually the percentage of cases which are fatal), or the case-fatality rate of a disease. This rate, however, may be affected in considerable measure by non-specific conditions which affect the host population, such as starvation, lack of proper medical care, secondary invasion by other micro-organisms, and similar factors. A more valid index of potential pathogenicity, although more difficult to estimate, is the proportion of infections which are clinically recognizable, or the ratio between clinical and subclinical infections. Each species of microparasite has a characteristic range in this respect. For example, an attack of pertussis confers a durable immunity against a subsequent attack. In the



average American city, about 75 per cent (Collins, 1929) of adults give a history of having had a clinically recognized attack of the disease, and more than 95 per cent of adults are immune. Thus, it would appear that perhaps one out of every five or six adults have acquired immunity by a subclinical infection.

A single attack of diphtheria likewise confers upon most individuals immunity against subsequent illness from the same cause. On the basis of data collected in the United States in 1929 (Collins, 1929), about 10 per cent of adults gave a history of having had the disease. Surveys conducted in typical communities at this time indicated that approximately 60 per cent of adults had acquired immunity to diphtheria, as evidenced by a negative Schick reaction. It appeared, therefore, that perhaps five out of six individuals had gained their immunity through subclinical attack. The ratio of clinical to subclinical attacks is not fixed precisely. It varies within a limited range for each infectious disease in relation to age, race, biologic qualities of prevalent strains of the specific microparasite and other factors (Frost, 1928).

Recent studies have revealed that about three out of four infections with *Coccidioides immitis* are subclinical and are identified only by change in reaction of the skin to coccidioidin. Only 20 to 35 per cent of infections are clinically recognizable. The manifestations of the disease vary from mild symptoms rarely diagnosed, to the full-blown characteristic syndrome known as San Joaquin or Valley Fever, with malaise, chills, fever, pleural pain, cough and headache, lasting a week or more. In an occasional case, perhaps 1 in 500, the disease goes on to progressive dissemination (coccidioidal granuloma) with a case-fatality of 30 to 60 per cent. A decade ago the latter was the only recognized form of the disease. (Smith et al., 1946).

## SUCCESSFUL PARASITISM

Successful adaptation of a species of microparasites to the human host does not imply a high order of pathogenicity. Rather the contrary is true. Success for a parasite as for any other living organism, can only be measured by the size of the population of its kind and its ability to survive and maintain these numbers in a constantly changing natural universe. There is no advantage if its host sickens and dies, since dissemination of its progeny accordingly becomes limited and soon ceases. The opportunities for scatter and chance of productive contact are increased in proportion to the length of time it can continue to multiply and find easy egress in large numbers from a host which is ambulatory and gregarious. Accordingly, a high case-fatality rate may be a disadvantage to survival of a parasitic agent. Conversely, a low ratio of clinical to subclinical infections and a long duration of the infectious period tends to insure wide dissemination. The microparasites best adapted for survival are those which cause infection with the least inconvenience and injury to the host, and create only a low-grade immunity of short duration. For example, the *Shigella* organisms which cause bacillary dysentery are much better adapted to survival in human populations than are the vibrios which cause cholera, although both diseases are transmitted in much the same manner. The cholera vibrio produces a violent host reaction of brief duration accompanied by a high mortality rate. It has been able to propagate continuously in human populations only in a very limited geographic area centered about India, where conditions of crowded living at a primitive level of sanitation and other factors are favorable. From these endemic centers, it has from time to time spread widely along routes of travel to other countries, prevailed for short periods, and then died out. Although introduced into North America several times during the

nineteenth century, it failed to become established in this continent, and, with the level of sanitation which has now been attained, it is highly improbable that it will. On the other hand, *Shigella* organisms cause a relatively mild host reaction, with a low-case-fatality rate, and are able to persist in the mucous membranes of the lower intestinal tract for longer periods of time. Bacillary dysentery is widely distributed throughout the world, in populations living under all kinds of conditions, and is still encountered occasionally in communities which maintain a relatively high level of sanitation.

### COMMUNITY SUSCEPTIBILITY

The pathogenicity of a specific species of microparasite implies a reciprocal range of resistance to infection on the part of the host species. The infection pattern is in general determined by the balance between the devices of aggression of the former and the mechanisms of defense of the latter, capacities for both of which are genetically transmitted. A human community is made up of a number of individuals who vary not only in their genetic capacity to react, but nearly always in previous experience with the predominant strains of the particular species of microparasite or its close relatives. Some individuals have acquired a complete immunity, some a partial immunity, some none. The proportion of a population at any one time which has little or no immunity determines the theoretic susceptibility status or the mass susceptibility of a community for the infectious disease which a specific microparasite causes. If a micro-organism is commonly prevalent in a community, the proportion is a constantly changing one as susceptibles are infected, develop immunity, and recover. If the immunity conferred by an infection is durable, then susceptibility decreases with age, and the age distribution of cases is consequently that of a "children's" disease. If the immunity conferred is temporary, as with many acute

respiratory infections, the same individual may be reinfected and consequently the disease attacks all ages, adults and old people as well as infants and children. Thus, community susceptibility has an age distribution which is specific for each infectious agent, the range of which is indicated by the age distribution of cases of the disease which it causes, provided of course that the infection is so widely disseminated that all classes of the population are exposed.

### INFECTIVE DOSAGE

Susceptibility is relative not only to the potential pathogenicity but also to the dosage of a specific strain of micro-organism. The attack rate of typhoid fever in a population exposed to a polluted city water supply is extremely low in comparison with that experienced by the same population exposed to food contaminated by a carrier. Further in the text, evidence is presented that the attack rate was higher among those directly exposed to food contaminated with type 5 beta-hemolytic streptococci than among their associates who were secondarily exposed through personal contact. Both epidemiologic and experimental observations support the concept that the size of the infective particle makes considerable difference in the outcome of exposure to air-borne pathogenic micro-organisms. For example, it is possible (Wells, 1948) that inhalation of a few tubercle bacilli in the nuclei of droplets coughed or sneezed into the atmosphere is of greater consequence than far larger numbers of organisms in coarse sputum particles which are strained out in the upper respiratory passages and pass down the alimentary tract instead of into the tracheal tree.

### CONTACT RATE

The qualitative variation taking place within the microparasitic and host populations, and the variations in conditions



which affect their interrelationships with each other and with the environment, results in quantitative changes. The size of the microparasitic population depends upon the rapidity of passage from person to person and the accumulated proportion of persons harboring the infectious agent at any one time. This is determined not only by the proportion of susceptibles but by the opportunities for progressive transfer to new hosts, i.e., the exposure or contact rate. This rate is affected by a variety of conditions, depending upon the requirements for transmission. For those diseases which are transmitted from person to person by some form of direct or indirect contact, the importance of the degree of crowding, or density of population, as determined by living in urban or rural areas, in private homes, in institutions, or in military installations is obvious. For those diseases which are transmitted to some extent at least by fecal contamination of food, milk or water, the importance of environmental sanitation and home hygiene is evident. For respiratory diseases transmitted to some extent by airborne particles—droplets, droplet nuclei and contaminated dust—sanitary ventilation of enclosed spaces is of importance (Subcommittee for the Evaluation of Methods to Control Air-Borne Infections, 1947). For diseases transmitted by insects there are a whole series of conditions which affect the numbers of the vector species, their access to man, and the requirements of the microparasite for completing a cycle of development. In every community these factors are constantly changing with the habits of the people, day in and day out, from season to season, and from year to year.

#### OPERATION OF CHANCE

If an individual in the infectious stage of the disease arrives in a community from which the disease has been absent for some time, what happens will be determined in

part by the susceptibility status, in part by exposure or contact rates, and finally by the operation of chance. For example, a person may develop measles and, since by chance the contacts immediately exposed are immune, no secondary cases will occur. Or a second and third case may occur without further transmission of the disease to susceptibles. So the chain of propagation of an infectious agent may build up or diminish and disappear, depending, on the one hand, upon the continuing chance contacts between cases and susceptibles and, on the other, upon contacts between cases and immunes.

#### PREVALENCE

The forces which create the dynamic biologic phenomena of infectious disease are, in the ultimate analysis, population pressures, i.e., the innate impulse of living microorganisms to multiply and survive by parasitism upon *homo sapiens*, and intelligently directed efforts of the host species to preserve its own integrity. The balance between these two forces is constantly fluctuating, just as are the interactions between other living species, as, for example, between the carnivores and their herbivorous food sources. When the equilibrium is a relatively stable one, it is manifested by an "endemic" prevalence. When the equilibrium is subject to sudden and violent disturbances, it is manifested by "epidemics." If the balance is in favor of the host, the disease shows a downward trend and tends to disappear. If the balance is in favor of the microparasite, the disease tends to increase in prevalence, and may, in certain instances, act as a human population check.

To facilitate reasoning it is necessary to express these phenomena in quantitative terms. The basic elements of this statistical methodology are formulae which represent prevalence or incidence. They are derived from the following schematic generalization:

$$\frac{\text{Numerator}}{\text{Denominator}} = \frac{\text{No. of parasitic population}}{\text{No. of host population}}$$

$$= \frac{P}{H} \text{ in a specified time and place}$$

The denominator (number of individuals in the host population) can in many situations be counted or estimated with considerable accuracy. The numerator (number of parasitic micro-organisms) can only be indirectly represented. It is correlated in a rough way with the number of deaths, or cases, or infections caused by a microparasitic population. These three indices are therefore available:

A death rate or mortality rate

$$\frac{\left\{ \begin{array}{l} \text{Number of deaths due to} \\ \text{specific disease} \end{array} \right\}}{\text{Number of population}} \times 100 *$$

A case rate, attack rate or morbidity rate

$$\frac{\text{Number of cases of specific disease}}{\text{Number of population}} \times 100 *$$

An infection rate

$$\frac{\left\{ \begin{array}{l} \text{Number of individuals harboring} \\ \text{specific microparasite} \end{array} \right\}}{\text{Number of population}} \times 100 *$$

Obviously, each of the three types of rate has its own implications. The one used will depend upon the questions to be answered and the availability of statistical information for the population group or groups under consideration. All are subject to errors of diagnosis and completeness of counting. Basic to effective use in reasoning is an assessment of the approximate validity of a rate. This can be done only when the accompanying text contains a clear statement of the universe of observation (denominator) in place or area, persons and time, the methods by which the deaths, cases or

infections (numerator) were discovered and recorded, and the clinical and laboratory criteria employed in diagnosis and classification. The soundness of inferences drawn from biostatistical material can never exceed the level of accuracy of the original data.

## INCIDENCE

To represent the shift in balance or changes in equilibrium between a micro-parasitic and a host population, it is necessary to show what happens in successive periods of time. For adequate expression, the numerator of the fraction then should preferably be the number of new cases or new infections which are reported to have their onset, or are discovered, or are admitted to a clinic in successive days, weeks, months or years. If the number of host population (denominator) remains relatively stable during the period under observation, the number of new cases or new infections alone will suffice to indicate the course of events without calculating rates. Thus, the incidence of disease, or an incidence rate, is a dynamic concept. It reflects changes in the frequency with which the microparasite is spreading and gaining access to new susceptible individuals, and, accordingly, the increase or decrease in microparasitic population.

An accurate statement of incidence must take into account not only the number of new cases (numerator) but the total number of new individuals at risk (denominator) in each successive time period. It makes a great deal of difference whether it is a closed or an open universe, i.e., whether the population is composed of the same, or approximately the same, individuals throughout the period of observation or whether the individuals in the population are changing through immigration and emigration. For example, an incidence of cerebrospinal meningitis in one army camp ten times greater than in another when expressed on the basis

\* This rate may be expressed on the basis of any population unit considered to be appropriate: per cent, per 1,000, per 10,000, per 100,000. The time unit chosen, whether it be hours, days, weeks, months or years, is also varied according to circumstances.



of "cases per thousand strength per year," may be due to the fact that in the latter the personnel is permanent, while in the former it is periodically changing through the arrival of recruits and the departure of graduates from a course of training, so that ten times as many individuals are at risk of infection during the course of a year.

### EPIDEMICS OF SHORT DURATION (SHARP OUTBREAKS)

The word epidemic is most commonly used to refer to the sudden or unusual appearance and/or temporary increase in incidence of a disease previously absent or occurring only sporadically in a small population group and limited environment. It is conventionally represented in a graph by plotting the number of cases (ordinates) by date of report or onset according to the selected time intervals, hours, days, weeks, (abscissae). The numbers usually show a regular ratio of increase in successive intervals to reach a maximum and pass over into a similar ratio of decrease so as to describe a more or less symmetrical curve, as illustrated in Chart 16.

It is to be noted that the span of time between the minimum and maximum incubation periods varies widely in different diseases. For example, in food poisoning due to staphylococcus toxin it is a matter of from 1 to 8 hours; in influenza, from 1 to 2 days; in measles, from 12 to 16 days; in homologous serum jaundice, from 2 to 6 months. By comparison of this span of time of the disease involved with the period during which the cases included in the outbreak have their onsets, an important inference can be drawn. If the onsets of all or nearly all the cases fall within an interval no greater than that of the known variation in incubation periods, then it can be assumed that they arose from a nearly simultaneous exposure to a common medium of dissemination or to a single source. Or, reversing the procedure, if it be known that the group

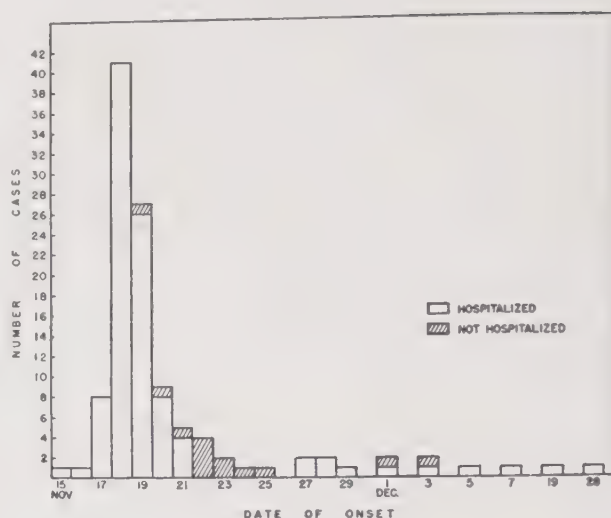


CHART 16. Distribution of cases of type 5 streptococcus infection by date of onset of symptoms. (Commission on Acute Respiratory Diseases, 1945, A Study of Food-Borne Epidemic of Tonsillitis and Pharyngitis due to B-Hemolytic Streptococcus, Type 5. Bulletin of the Johns Hopkins Hospital, 77, 143-210.)

of persons selected by a disease have been together upon only a single occasion, then the common exposure must have occurred at this time and the variation in the incubation periods of different individuals can be calculated.

These considerations may be illustrated by an epidemic of tonsillitis and pharyngitis due to  $\beta$ -hemolytic streptococcus, type 5, in the members of two companies of an airborne infantry regiment at Fort Bragg. The following account is paraphrased from the report of The Commission on Acute Respiratory Diseases, Fort Bragg, North Carolina (1945).

The chronological sequence of development of the epidemic is illustrated in Charts 16 and 17; the former indicates the date of onset of symptoms of hospitalized and nonhospitalized cases; \* the latter the date of discovery of healthy carriers.†

\* Nonhospitalized cases represented subjects who made dispensary visits for symptoms referable to the respiratory tract within a week after Type 5 Streptococci were first isolated in their throat cultures.

† Editorially defined as subclinical or inapparent infections.

### Primary and Secondary Attack Rates.

The definitions of the terms "primary" and "secondary" were, of necessity, arbitrary but were made after consideration of all the facts elicited in regard to the time of original infection and incubation period. Primary cases were defined as subjects who became ill and harbored Type 5 Streptococci at the time of the first culture survey on November 20. Chart 17 indicates that more than 75 per cent of the total number of cases had an onset of symptoms on or before that time. Epidemiologic evidence dated the time of primary in-

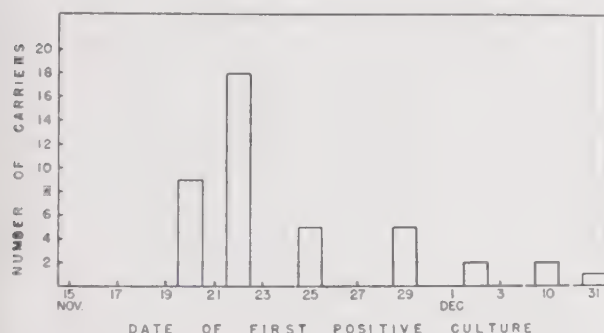


CHART 17. Distribution of healthy carriers of type 5 streptococci by date of first positive culture. (Commission on Acute Respiratory Diseases, 1945, A Study of Food-Borne Epidemic of Tonsillitis and Pharyngitis due to B-Hemolytic Streptococcus, Type 5. Bulletin of the Johns Hopkins Hospital, 77, 143-210.)

fection as the morning of November 17. Two cases had a clinical onset before November 17; this discrepancy may have been due to an error in history taking or to the presence of respiratory symptoms due to other causes antedating the infection with Type 5 Streptococci. Secondary cases were defined as those who did not harbor Type 5 Streptococci on November 20 or before but who subsequently became ill and acquired the organisms. "Early" secondary cases were those who acquired the organisms before November 27, and "late" secondary cases those who first harbored the organisms after that date. Healthy carriers were classified by the same cultural criteria as cases.

Since 27 men had already become ill by noon of November 18, there were numerous foci of infection scattered throughout the group for 48 hours before the first culture survey was made. It is therefore entirely possible that some of the cases and carriers classi-

fied as "primary" may, in fact, have been infected secondarily by contact in the barracks with earlier cases or carriers. However, the separation made of primary and secondary cases and carriers seemed the most reasonable with the data available.

Primary and secondary attack rates were determined on the group of 228 enlisted men in G and Hq Companies who were present on November 17 and remained under observation until December 31. During this period all hospital admissions were studied, dispensary visits counted, and 6 periodic culture surveys made. The primary attack rate for this group was 41.7 per cent (Table 54). Of these, 86 were primary cases, and 9 carriers. The secondary attack rate, among those exposed to risk, was 30.1 per cent, of which somewhat less than one-half were secondary cases and slightly more than one-half were secondary carriers. In the course of 6 weeks from the beginning of the outbreak, 59.2 per cent of the men in the group acquired Type 5 Streptococci; 45.6 per cent were cases, and 13.6 per cent carriers.

The case-to-carrier ratio of the primary infections was approximately 10 to 1, which suggested a massive dose of infection and indicated that almost all subjects who acquired the infectious agent at the time of the original seeding became clinically ill. Among the secondary infections, the case-to-carrier ratio was approximately 1 to 1. More than two-thirds of the healthy carriers appeared to acquire the organisms as a result of secondary contact spread.

**Source of Infection.** Because of the explosive nature of the epidemic and the concentration of cases with onsets on November 18 and 19, a common source of infection was immediately suggested. The further evidence of localization of the cases entirely to two companies served by a common mess and the absence of infection in an adjacent company served by a separate mess, pointed strongly to food as the vehicle of infection. From a consideration of the time of onset of illness and the probability of an incubation period of not more than 3 or 4 days, the search for the source of infection was concentrated on foods served at mess on November 16 or 17.

Investigation indicated that the milk supply could not have been infected unless it was contaminated after delivery to the mess hall. The milk was pasteurized and delivered in quart bottles from a large dairy which regularly served more than 10,000 troops on the Post.



TABLE 54. PRIMARY AND SECONDARY ATTACK RATES IN 228 ENLISTED MEN IN G AND HQ COMPANIES

TYPE OF INFECTION	STRENGTH EXPOSED TO RISK	NUMBER			PER CENT		
		CASES	CARRIERS	TOTAL	CASES	CARRIERS	TOTAL
Primary.....	228	86	9	95	37.7	3.9	41.7
Secondary:							
Early.....	133	11	13	24	8.3	9.8	18.0
Late.....	109	7	9	16	6.4	8.3	14.7
Total.....	228	104	31	135	45.6	13.6	59.2

On November 23 the enlisted personnel and officers were questioned regarding specific items of food eaten on November 16 and 17. Infection rates were calculated according to the history of eating specified foods, the infected individuals being those who harbored Type 5 Streptococci on the cultural survey conducted on November 20 (Table 55). Infection rates among those who gave a definite history of eating or not eating the particular items of food revealed a significant difference only for creamed eggs which were served for breakfast on November 17. Seventy-nine per cent of those who said they had eaten the eggs were infected, whereas only 35 per cent of those not eating eggs had positive cultures on November 20. The probability that this

difference was the result of chance alone is 1 in 100,000. The source of infection of the 22 men who said they had not eaten creamed eggs could not be determined. The dietary interviews were conducted six days after the creamed eggs were served and were therefore subject to considerable error. It is also possible that some of these 22 cases may have been early secondary infections. At the time the investigation was made none of the food served before November 20 was available for bacteriologic examinations. The exact manner in which the creamed eggs were prepared was not ascertained because of conflicting testimony. It was established, however, that the eggs had been boiled and then sliced by hand at least 10 hours before serving. None of the

TABLE 55. RELATION OF PRESENCE OR ABSENCE OF TYPE 5 STREPTOCOCCI IN THROAT CULTURES ON NOVEMBER 20 TO HISTORY OF EATING SPECIFIED FOODS ON NOVEMBER 16 AND 17

FOOD	TOTAL WITH FOOD HISTORY KNOWN		TYPE 5 STREPTOCOCCI				P *
			NUMBER		PER CENT		
	ATE	DID NOT EAT	ATE	DID NOT EAT	ATE	DID NOT EAT	
Spareribs . . . . .	129	9	73	4	57	44	0.47
Fruit salad . . . . .	127	10	72	5	57	50	0.69
Creamed eggs . . . . .	63	62	50	22	79	35	0.00001
Cauliflower . . . . .	43	85	27	46	63	54	0.35
Noodles . . . . .	95	31	59	15	62	48	0.16

\* Probability that differences as great as these could arise from sampling.

cooks harbored Type 5 Streptococci on November 20, nor was evidence obtained by examination or history of recent infection in any of them.

The investigation of an explosive outbreak may be relatively simple, since a priori one is concerned only with discovering the common factor. A microparasitic population has suddenly found an opportunity and a medium by which it can be disseminated to a group of host individuals in a short space of time. A certain proportion of the exposed group are susceptible and to that extent they come down with clinical or subclinical attacks characteristic of the specific infectious agent. The problem is resolved into discovering upon what common occasion, or by what common medium, the persons so selected could have had a more or less simultaneous exposure.

If the portal of entry of the specific micro-parasite involved is or may be through the alimentary tract, attention is then centered upon articles of food or drink, particularly water supply, milk supply or food that has been insufficiently cooked or which has been allowed to stand several hours after preparation in a warm place, allowing opportunity for growth of the pathogenic micro-organisms. The remainder of the investigation is then directed toward elucidating the conditions which permitted the contamination to occur, with the practical objective of instituting appropriate preventive measures.

### EPIDEMICS OF LONG DURATION (PROGRESSIVE EPIDEMICS)

When the span of time of an epidemic wave is much greater than the average incubation period of the particular disease in question, then it can be assumed: (1) that exposure to dissemination by a common medium has been prolonged, or (2) that the infection is being propagated by progressive host-to-host transfer ("contact contamination") or (3) that there is a combination of common-medium dissemination with sec-

ondary contact transmission. An epidemic which is principally or solely the result of progressive host transfer is frequently called a "progressive" epidemic.

In considering the prevalence in large population units over long periods of time, months or years, the term epidemic may refer only to a peak in the oscillating incidence of a disease. How great the increase must be before it is regarded as "epidemic" is a matter of judgment and is influenced by psychologic attitudes. The greater the fear of a disease, or the more unusual it is in a community, the smaller the increase needed to justify use of the descriptive term. Many statistical devices have been suggested for making the definition more objective and precise (Bundesen and Hedrich, 1925; Rich and Terry, 1946), but none has yet received general sanction. Dependence is placed upon comparing the current incidence of each specific disease with its previous incidence in the same population group and at the same time of the year. This "expected number" or "norm" is commonly expressed as the average or median of the experience of the three, five or seven preceding years by weeks or months. When this average or median is exceeded in several successive time periods, the disease shows a tendency which, if sustained and great enough, merits a pronouncement as being epidemic.

Each infectious disease has a seasonal variation which follows a more or less regular pattern, reaching a maximum distribution about the same time each calendar year, when conditions are most favorable for transmission. Each is subject also to an interannual variation or secular trend which may show slight or wide fluctuations. Some diseases manifest a cycle of periodicity, epidemic years occurring at fairly regular intervals of two to three years, or perhaps four or five years or longer (Commission on Acute Respiratory Diseases, 1946a). Others range from being rather constant in their endemic level, as, for example,



tuberculosis, to being relatively unpredictable in their annual behavior, as, for example, pneumococcus pneumonia.

Chart 18 illustrates the manner in which the prevalence of meningococcus meningitis has varied in the United States during the period from 1916 to 1944 (Gover and Jack-

son, 1946). The word "epidemic" may perhaps be used to describe the periods of high prevalence, even though they are of several years' duration. It is apparent from the chart that there have been four such periods, reaching maximum rates in 1916, 1929, 1936 and 1943. The number of years included in each "epidemic period" is obviously a matter of arbitrary decision and depends upon the definition of excessive prevalence which is employed.

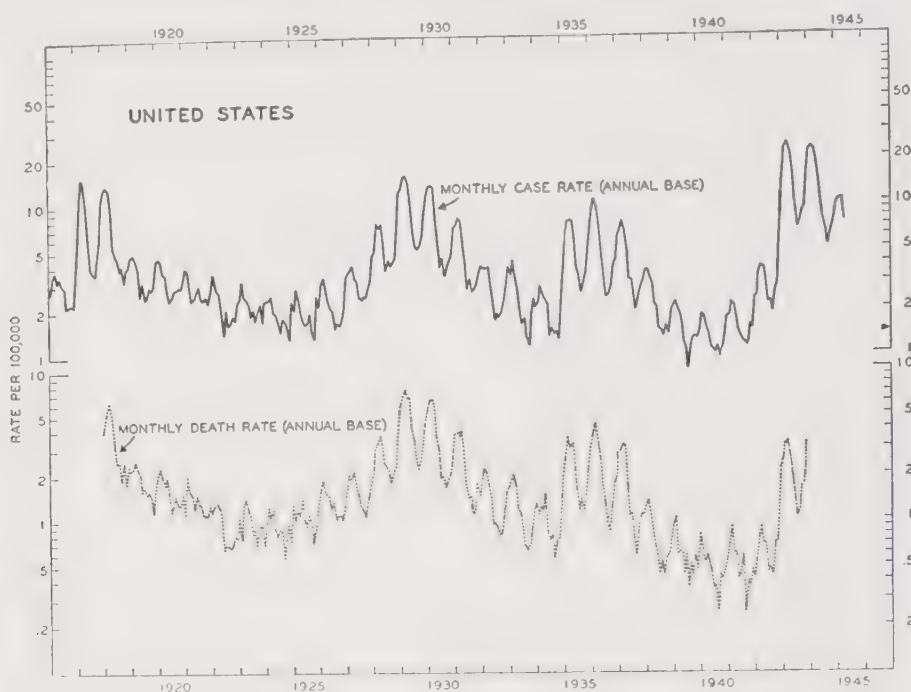


CHART 18. Monthly morbidity and mortality (annual base) from cerebrospinal meningitis in the United States from 1916 to 1944. Deaths are for cerebrospinal (meningococcus) meningitis. (Gover, M., and Jackson, G., 1946, Cerebrospinal meningitis. A chronological record of reported cases and deaths. Pub. Health Rep., 61, 440.)

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### EPIDEMIC THEORY

From the epidemiologic point of view, the simplest of all infectious diseases is measles. Table 56 illustrates the manner in which its

related with the season of the year, increasing to a maximum in the spring and decreasing to a minimum in the summer months. The time at which the maximum incidence is reached in each year varies within fairly wide limits. In some years, the total incidence is relatively low, in others it rises to a level regarded as epidemic. These epidemic years appear to recur at fairly regular intervals in the same locality.

A century ago the periodicity of measles epidemics was known and discussed (Hirsch, 1883). The causes were thought to be obscure and complex, although it was generally accepted that the accumulation of susceptibles was an important factor. A more precise numerical approach to the explana-

TABLE 56. MEASLES CASES BY MONTHS IN PROVIDENCE FROM 1917 TO 1940 \*

YEAR	JAN.	FEB.	MAR.	APR.	MAY	JUNE	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.	TOTAL
1917	33	47	62	109	119	36	13	7	2	1	8	55	492
1918	55	98	373	1,232	1,299	780	261	23	8	6	5	3	4,143
1919	1	4	4	4	5	4	3	3	1	2	1	3	35
1920	125	127	136	279	404	288	146	38	45	53	190	191	2,022
1921	329	585	665	390	266	99	28	10	1	2	7	26	2,408
1922	89	4	3	26	25	22	23	19	7	16	131	652	1,017
1923	680	1,228	1,470	687	383	117	29	6	3	10	7	7	4,627
1924	5	6	3	11	16	30	15	2	2	1	5	2	98
1925	13	11	6	15	18	30	58	50	13	81	417	1,224	1,936
1926	2,057	1,360	648	348	196	105	48	8	1	0	0	4	4,775
1927	5	2	1	1	2	2	6	2	0	9	7	23	60
1928	45	112	422	1,081	883	800	508	77	18	36	36	61	4,079
1929	84	189	261	399	276	111	38	4	3	2	0	0	1,367
1930	2	0	1	4	23	46	22	8	1	0	2	0	109
1931	1	2	49	158	456	358	179	99	22	191	337	1,548	3,400
1932	2,799	2,037	574	199	81	11	2	0	0	0	0	0	5,703
1933	0	0	0	3	3	6	5	2	4	0	1	1	25
1934	4	11	21	18	29	106	44	25	8	5	1	7	279
1935	13	57	343	1,351	1,953	1,279	241	17	4	1	0	48	5,307
1936	119	74	92	76	83	17	11	4	0	0	9	77	562
1937	422	811	1,184	711	472	129	31	4	0	2	3	3	3,772
1938	2	5	4	2	0	0	0	3	1	0	0	3	20
1939	33	35	40	118	317	286	157	64	20	89	267	446	1,872
1940	569	495	530	462	543	372	121	20	1	0	1	1	3,115
Total	7,485	7,300	6,890	7,684	7,852	4,934	1,989	495	165	507	1,435	4,385	51,221

Epidemics culminate in May, 1918; March, 1921; March, 1923; January, 1926; April, 1928; January, 1932; May, 1935; March, 1937; March(?), 1940. In this period of 262 months there are 9 major peaks, but we must not count both ends. The average time between peaks is  $33 \pm 7.9$  months, not 2 years. For the mean we write  $33 \pm 2.8$  months. In Glasgow we estimate 40 months between peaks from 1888 to 1927, incl., based on Soper's data [J. Roy. Statist. Soc., London, 92, 34-61 (1929)]. How many peaks one counts depends on the interpretation one gives to the qualifying adjective "major" and what allowance one makes for seasonal interruption of an epidemic.

\* Adapted from Wilson, E. B., and Burke, M. H., 1942, The epidemic curve, II. Proceedings of the National Academy of Sciences, 92, 43-48.

tion of periodicity of measles began with the contribution of Sir William Hamer (1906). Following his lead, a biometrician (Soper, 1929) in the course of an examination of possible methods of forecasting common contagious diseases "was led to adopt the simplest mathematical postulate that would describe on a first measure the generally accepted mechanism of epidemic measles, if the accumulation of susceptibles were really the prime factor, to compare the deduced results with the observed facts and then modify the primary hypothesis." Soper's work in turn stimulated W. H. Frost (unpublished). Lowell J. Reed (unpub-

lished), A. W. Hedrich (1933), A. G. McKendrick (1940) and E. B. Wilson and M. H. Burke (1943) to elaborate the statistical approach to epidemic theory. This has elucidated quantitatively the relationships of the principal factors involved, and contributed to a rational explanation of the epidemiologic behavior of measles.

The fundamental facts with which we start are simple. The biologic attributes of the measles virus and the requirements for infective transmission from case to susceptible remain relatively constant. The dynamics of the mass reaction are due to the flow of the virus through the human popu-



lation. Each new case is due to effective contact with a preceding case in the infectious stage. Susceptibles effectively exposed to cases become cases in the next time period; cases recovering from the infection accumulate as immunes. The susceptibles are being constantly recruited through births and immigrants, and depleted through becoming cases and immunes, or through deaths and emigration.

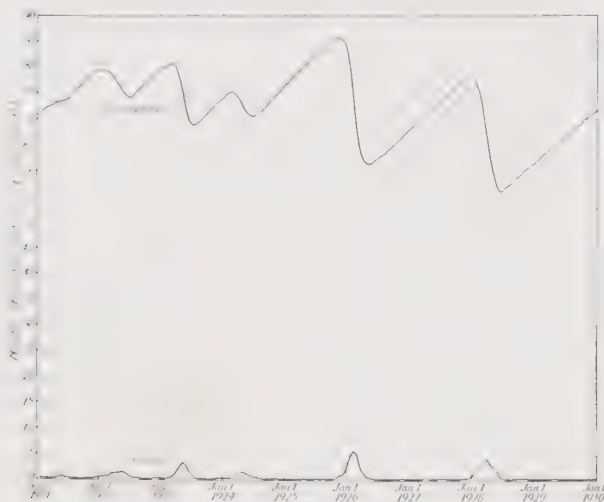


CHART 19. Estimated cases of measles and susceptibles in the Baltimore population under 15 years of age.

Upon the basis of a series of logical and reasonable approximations and assumptions, Hedrich (1933) made monthly estimates of the child population susceptible to measles in Baltimore from 1900 to 1931. As shown in Chart 19, during a 9-year portion of this period the calculated proportion of susceptibles in the population under age 15 did not rise above 53 per cent nor fall below 32 per cent. The percentage figures are only approximations, but the implications are significant. When the proportion of susceptibles was low, the incidence of measles tended to be low; consequently, susceptibles accumulated. When the proportion of accumulated susceptibles approached what McKendrick calls a "threshold density," the situation was favorable for the support of an accelerated incidence of cases,

or an epidemic. During a short period of time, the proportion of susceptibles fell rapidly as they became cases and subsequently immunes. As the proportion of immunes increased, more and more cases failed by chance to make effective contact with susceptibles and the incidence of new cases fell accordingly.

It is apparent, therefore, that the principal factor determining the occurrence of progressive epidemics of measles is the proportion of susceptibles in the population at risk, and that the termination of the epidemic wave is due to the dampening effect of the cumulation of immunes and not necessarily to the exhaustion of susceptibles, since many escape effective exposure. The proportion of susceptibles required to support an epidemic, and per contra the post-epidemic proportion remaining, will vary in every community, and even in the same community at different times of the year according to the "contact rate." It is much easier to start an epidemic spread of the disease during the winter than in the summer, due to changes in the "contact rate," which are only partially understood.

By utilizing the simplified premises in measles, and representing the four principal factors by appropriate symbols, it is possible to derive a dynamic equation by which, given (1) the number of cases, (2) the number of susceptibles, (3) the number of the total population, and (4) assuming an arbitrary value for the "contact rates" in one time period of 14 days, the number of new cases which will arise in the successive time periods of the same length can be calculated. Departures from the course of epidemics predicted from the equation can be explained as due to the operation of chance. In the community where sufficient data are available, the correspondence between cases predicted by such a formula and the cases observed is reasonably good within certain limits. The same kind of reasoning and mathematical postulations can be applied to other infectious diseases, but the factors

which must go into the equation become more complex and we are unable to obtain numerical values for them from observations made in nature. The practical usefulness of the statistical approach to epidemic theory becomes correspondingly limited.

### EXPERIMENTAL EPIDEMIOLOGY

Another approach to the discovery of laws or general principles governing the behavior of infectious diseases in human populations is through observations made upon epidemics in experimental animal colonies. Notable among the many contributions are those made upon colonies of white mice by Theiler (1941) and Traub (1939) on virus infections of the central nervous system; by Webster (1932, 1946) and his associates on salmonella, pasteurella, pneumococcus and Friedländer bacillus infections; by Topley and his associates (Greenwood et al., 1936; Topley, 1942) on salmonella, pasteurella and ectromelia virus infections. These studies are too extensive to permit detailed review. It will be useful perhaps to comment briefly upon the methods used and the knowledge gained by the latter two groups of investigators mentioned above.

The general procedure was to assemble uninfected animals in unit cages whose arrangement could be altered so as to simulate a community of any desired size. A constant regime of cleaning and feeding was established, and appropriate measures taken to prevent the introduction of extraneous pathogenic micro-organisms. An epidemic was started by introducing into an uninfected animal colony a certain number of animals infected with the microparasite selected for the experiment. The course of the subsequent epidemic was indicated by the occurrence of specific deaths, proved by autopsy and culture. Effort was made to hold all the important factors constant except the one under examination, and to note

the effect this variable had upon the course of an artificially produced epidemic.

It became evident very early in this work that a constant genetic stock of experimental animals was fundamental to control of the host variable. As had long been known to the plant pathologists, it was found possible within certain limits by selective mating to breed out lines which were relatively resistant or relatively susceptible to infection with a particular micro-organism. It was demonstrated, for example, that there may be selected promptly from a hybrid stock of mice, of which 40 or 50 per cent die, lines in which as high as 95 per cent and as low as 15 per cent succumb following a standard dose of *B. enteritidis*. This afforded experimental support for the concept of innate differences in resistance to a particular microparasite genetically transmitted in human families, lines of descent, or races, a phenomenon well illustrated in human experience, for example, by the differences in the host reaction of the white and Negro race to infection with *Mycobacterium tuberculosis*.

The possible importance of nutrition of the host to natural resistance to infection was appreciated. If a diet were so poor in quality or quantity as to bring about a state of debility, experimental animals whose lives were already jeopardized from the consequences of produced deficiency would have a higher death rate than well-nourished animals if subjected to the added insult of infection. Obviously, it was desirable to hold this factor constant by providing a uniform and well-balanced diet in the test and control groups. It was noted, however, that a diet which was well balanced for normal growth and development, was not necessarily well balanced in its effect upon host resistance to infection with a specific micro-organism. This question has been explored extensively by many investigators in relation to various infections experimentally produced in animals. The studies of Schneider and Webster (1945) on the effect



of diet on the response of several genotypes of white mice to *Salmonella* infections, is a particularly valuable contribution. Resistance-promoting food elements were apparently of very limited importance as one of the factors affecting the results of experimental epidemics produced by *Salmonella enteritidis*.

The variability in the biologic potentialities of the strains of infecting micro-organisms employed received considerable attention. A theory had been advanced by certain speculative epidemiologists that the rise of a progressive epidemic is principally, if not wholly, due to a progressive increase in "virulence" of the specific agent, with the rapid human passage. As the infectious agent encounters more resistant individuals there is a progressive decrease in "virulence" resulting in less frequent passage and a falling off of the number of new cases in successive time periods. To test this theory, methods were devised by Webster for measuring the "virulence" of a specific strain of micro-organism for groups of mice by administering a fixed dosage. In artificially produced epidemics, sample cultures were obtained from animals dying at various stages. Comparative titrations were made on strains from epidemics of pasteurellosis in rabbits, chickens and mice. Similar titrations of two serologic types were made during the course of mouse typhoid infections in mouse populations. A total of 300 or 400 titrations were made under many conditions to test the theory of fluctuating virulence. "The results were invariably negative and showed a constancy and fixity of disease-producing power of a given strain of organisms under all conditions of natural infection. . . ."

From his experience with experimental epidemiology, Webster was inclined to believe that in all instances changes in biologic potentialities of specific microparasitic species are of little or no importance in determining the secular rise and fall of

epidemic waves. While this is undoubtedly true within certain limits and for many parasitic species, there may be some which are relatively more unstable and inclined to produce mutants. The possibility that bacterial and virus dissociation may occasionally play a role cannot be ignored (Zinsser and Wilson, 1932). The development of sulfadiazine-resistant or penicillin-resistant strains of bacteria is a pertinent indication of what may happen in nature.

In a series of experiments it was demonstrated that when infected animals were introduced in a closed universe of susceptible animals, the ensuing epidemic quickly subsided as susceptibles died or became immune, although a few escaped infection. An epidemic started in this manner could be maintained in an open universe if sufficient susceptible recruits were added at regular intervals. The course of the epidemic was modified by the relative number of susceptibles and the rate at which they were added. If the conditions were held relatively constant, the balance between the micro-parasitic and the host population tended to reach a stabilized equilibrium. This was violently disturbed by a major change in the contact rate which was accomplished by bringing a large number of animals previously dispersed in small single cages into a single colony in a large cage.

These and other experiments added support to some of the generalizations derived from experiences with epidemics in human populations under natural conditions. They emphasized particularly the accelerating effect upon incidence of an inflow of susceptibles into an infected community, and of aggregation of individuals into large groups (crowding), and, per contra, the dampening effect upon incidence of accumulation of immunes. But the actual quantitative importance of each of these factors varies with the disease, its mode of transmission, the host relationships involved, and the local circumstances.

## EVALUATION OF PREVENTIVE MEASURES

With most of the common infectious diseases knowledge has advanced to a point where the principal factors which determine incidence and distribution are generally recognized. With many, if not all, however, there is need for epidemiologic studies which will more exactly define these factors and establish their relative (crudely quantitative) importance. This statement, however, conceals an ignorance of the exact mechanism of transfer. Such questions as the following remain unanswered. To what extent is the virus conveyed more or less indirectly by droplet nuclei of contaminated dust in air currents? What is the size of the particle which, when inhaled, will reach the upper respiratory tract essential for infective contact? To what extent is the virus conveyed directly, i.e., by droplets from person to person in what might be called conversational proximity? To what extent is the virus conveyed by contact, i.e., by contamination of articles with infective secretions and transferred by hands to the mouth of a susceptible, etc.? The relative importance of these different routes of transmission must be evaluated if measures introduced to prevent spread are to be maximally effective, such as the use of ultraviolet light and germicidal mists, dust suppression, of surgical masks and goggles, and of aseptic nursing or isolation technics.

To put the thought in more general terms, it is necessary to effectiveness that measures of prevention be directed against those conditions which are of actual importance in the particular situation rather than against the much wider range of conditions which may possibly contribute to the prevalence of the disease. Innumerable instances could be cited in which public health campaigns or measures thought to be theoretically sound and rationally conceived failed

to accomplish the reduction which was expected.

## SECONDARY ATTACK RATE

A classic example of critical evaluation of measures to prevent the spread of common contagious diseases is afforded in the development and use of the secondary attack rate, with particular reference to scarlet fever and diphtheria, by Dr. Charles V. Chapin, for many years health officer of Providence, Rhode Island. It is related in some detail by W. H. Frost (1938) in a discussion of the familial aggregation of infectious diseases.

The principles and the applications of the method have the merit of yielding information which is easily understood and directly related to the practical problems of the health officer. The ultimate epidemiologic unit in a civil community is the family or household, a group of people, mostly of close kinship, sharing a common environment, living in close contact in a manner easily described, and usually under the eye of a single medical or lay observer. The degree of contagiousness of different diseases can be measured by a statistical index derived from familial experience. The first case to occur in a family is designated as a primary case. A census is made of the exposed members of the family, classified by age, sex or other conditions which it is desired to take into account, especially with regard to their past history of having had the specific disease in question or specific immunization against it. A record is then kept of cases occurring in any member of the household within time limits defined specifically for each disease with reference to the onset of the primary case, so as to include those probably infected by contact. It is then possible to summarize the observations on a large number of families and obtain an index of average experience based upon the ratio between secondary cases and



exposed persons, or exposed persons classified as to age, sex, relationship, previous history, immunity status or other quality. Schematically represented:

Secondary attack rate = 
$$\frac{\text{Number of secondary cases}}{\text{Number of exposed persons}} \times 100$$

Table 57 illustrates the manner in which the intrafamilial spread of different diseases can be compared.

that a practical objective of preventive medicine is to decrease the risk of the penalty of disease and death rather than of a subclinical immunizing infection.

While the secondary attack rate is a satisfactory device for evaluation of measures designed to reduce intrafamilial spread, it is only indirectly and by inference an indication of their effectiveness in reducing community spread. It is obvious that in dealing with a disease such as diphtheria it

TABLE 57. SECONDARY ATTACK RATES FOR POLIOMYELITIS, SCARLET FEVER AND DIPHTHERIA \*

AGE	POLIOMYELITIS NEW YORK CITY, 1916			SCARLET FEVER PROVIDENCE, R. I., 1904-09			DIPHTHERIA PROVIDENCE, R. I., 1904-13		
	NO. IN FAMILIES PRIMARY CASE EX- CLUDED	SUBSE- QUENT CASES	SUBSE- QUENT ATTACK RATE	NO. IN FAMILIES PRIMARY CASE EX- CLUDED	SUBSE- QUENT CASES	SUBSE- QUENT ATTACK RATE	NO. IN FAMILIES PRIMARY CASE EX- CLUDED	SUBSE- QUENT CASES	SUBSE- QUENT ATTACK RATE
0- 5	10,540	335	3.18	1,493	360	24.1	2,006	295	14.9
6-10	4,575	58	1.27	1,088	279	25.7	1,410	219	15.5
11-20	4,994	10	0.2	1,404	136	9.7	2,137	148	6.9
Over 20	17,191	4	0.02	4,339	52	1.2	7,529	136	1.8
Total	37,300	407	1.1	8,324	827	9.9	13,082	798	6.1

\* Adapted from the Hygienic Laboratory Bulletin, 1913, 90, 125 (Tables 59 and 60).

It is to be noted, however, that this index is based upon the frequency of secondary clinical cases following the occurrence of a primary clinical case. It does not take into consideration the spread by subclinical infections. It is useful nonetheless in answering certain questions: for example, (1) given a case of a communicable disease in the family, what is the risk of clinical attack borne by others in the same household within specified periods of time? (2) to what extent can risk of clinical attack be reduced by preventive measures, such as removal of the primary case to the hospital, immunization of exposed susceptibles? etc. It is pertinent to remark in this connection

might be possible to demonstrate that by prompt isolation of the primary case the secondary familial attack rate could be measurably lowered. Yet, if there are many individuals who have unrecognized subclinical infections for each individual who has a clinically recognized attack, and both categories are involved in maintaining passage from person to person, the effect of prompt isolation of cases upon the incidence in the community as a whole may be so small as not to be measurable.

The evaluation of preventive measures in reducing the incidence of a disease in a large population unit, such as a city, is fraught with difficulty. Allowance must be

made for the natural trend of the disease due to changes in the complex of factors other than the one or ones which are affected by the administrative measures. Occasionally, nature performs an experiment, which, if brought under adequate epidemiologic perception, answers a crucial question. A classic example, which should be read by every student of epidemiology, is presented in the observations made by John Snow (1865) on the relation of purification of water supplies to the incidence of cholera in different districts of London during the epidemic of 1854-55.

Nature, however, seldom sets the stage for a scientific experiment in such manner that it is possible to observe two population groups alike in all important respects except with regard to one factor. So it becomes necessary to set up such groups artificially if our many questions as to the effectiveness of control measures are to be answered. Unusual opportunities for such studies were afforded in military organizations during the war. The many considerations which must enter into investigations of this type are illustrated by a study of the effect of double bunking in barracks and of oiled floors and bedding on the incidence of respiratory disease in new recruits (Commission on Acute Respiratory Diseases and Commission on Air-Borne Infections, 1946).

## EVALUATION OF IMMUNIZATION AND CHEMOPROPHYLAXIS

The same kind of considerations enter into epidemiologic investigations designed to evaluate the prevention of a specific infectious disease by an immunization procedure or by the prophylactic administration of antibiotics or chemical compounds. The preliminary work in testing effectiveness and safety are carried out in the laboratory upon experimental animals. When sufficient evidence has been accumulated to justify the use, the final evaluation of the efficacy of such agents can only be obtained by

human trial. Furthermore, these observations must be so controlled as to merit scientific acceptance of results. Failure to meet this necessity has led in many instances in the past to the exploitation of biologic products and chemical substances which was unwarranted, and at times actually detrimental. It has become painfully evident that evaluation by "clinical impressions" is unreliable.

The basic requirements of critical trials upon human beings are well known, but the actual conduct of such an experiment is fraught with practical difficulties. Ideally, two groups of persons, a test and a control group, are placed under observation. They must be alike in all essential respects, particularly those which relate to their susceptibility at the beginning of the experiment, and their exposure to natural infection throughout the period of observation. The substance to be tested must be administered without discrimination, if possible alternate individuals receiving a placebo or blank. It is highly desirable that neither the subjects themselves nor the investigator who is responsible for their subsequent follow-up and observation should know who has received the test material and who has not. In this manner, errors due to unconscious human bias may be obviated. Individuals of both groups must be examined with equal frequency, care, and for equal periods, and for a sufficient length of time to insure an adequate test of the protection afforded. The criteria used in clinical diagnosis must be clearly stated. The resulting differences in attack rates in the two groups must be sufficiently large to be statistically significant.

This is a basic outline of the general approach to such problems. There are always many perplexing circumstances and occurrences tending to disturb the results for which allowance must be made in some manner. An illustration of this type of epidemiologic studies designed to evaluate critically an immunization procedure will



be found in Bell (1941) on pertussis prophylaxis with two doses of alum-precipitated vaccine. An example of a study planned to assess critically the value of prophylactic administration of a drug will be found in the report on the dynamics of meningococcal infections and the effect of chemotherapy by Phair and Schoenbach (1944).

### EXTRAHUMAN RESERVOIRS

Up to this point, for the sake of simplicity in discussion, attention has been focused upon infections which are transmissible directly from one individual to another of the same host species. These are due to microparasites which in the evolutionary process of host-wandering, mutation and selective adaptation have found conditions required for continuous survival fully satisfied by relationships established with this single host species and attendant environmental circumstances. Some of the most highly fatal and devastating diseases of man, however, are due to microparasites for whose continuous survival the human host bears little or no responsibility. He plays the role of a bystander and suffers accidentally from the struggle which is going on among other forms of life which are a part of the world in which he lives, or which he invades in competition for food and shelter. To understand and effectively interfere with the spread of disease in human communities due to microparasites which have

multiple host relationships, it is necessary to take into account in addition to the factors previously considered those which determine the distribution of the specific infectious process on a subhuman level. Indeed the epidemiologic manifestations of such a disease in human populations is very largely a reflection of the waxing and waning of the microparasitic population in its extrahuman reservoir of multiplication involving animals, birds or insects and the attendant ecology.

One function of epidemiology is to collect scattered observations upon the occurrence of such diseases in human population groups living and working under various circumstances, to search for the specific microparasite in animals, birds and insects with which patients may have been in contact, to study infections experimentally produced in suspected host species and, finally, to assemble all of the available evidence into a tentative pattern consistent with the facts.

With diseases due to bacteria which have multiple host relationships, such as *B. anthracis*, *Brucella melitensis*, *Pasteurella tularensis* and *Pasteurella pestis*, the principal facts determining their occurrence in human populations have been established. To a variable degree, however, there remain details, some of them important, which are yet to be elucidated. Studies of the ecology of plague (Meyer, 1942) afford a fascinating illustration of the multiplicity of factors involved.

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## 37

# The Cultivation and Identification of Pathogenic Bacteria

### INTRODUCTION

#### GENERAL PRINCIPLES AND THE RELATIONS OF LABORATORY AND WARD

Certain general principles are common to all infectious diseases although many variables make it necessary to study each type separately and even each case of infection presents its own particular problems. The first principle is that the most exact diagnosis is furnished by the isolation and identification of the recognized causative agent. Ideally, this requires the application of all available knowledge, but the usual pressure of work in the laboratory of necessity limits what can be undertaken for every case in a routine service. So it becomes essential to develop a scheme of procedure designed not to miss any well-known pathogenic micro-organism, and capable of being expanded to meet special circumstances and requirements. The isolation and identification procedure is not always possible since certain organisms cannot yet be grown or only with great difficulty and many another requires special materials and conditions. It is also prevented when the lesions are not accessible and when organisms are scarcely obtainable at particular stages of the disease. The scheme of procedure must therefore include means of recognizing antibodies

formed by the patient during the course of infection or free antigens liberated into the body fluids and tissues by the invading micro-organisms. This, too, has its limitations. Either the known applications of immunity reactions are not delicate enough or the reacting substances are only present in too minute quantity for detection. Above all, no laboratory can possibly maintain every possible requisite antigen or antibody necessary for this purpose. The upshot of the situation is that no laboratory can be expected to be ready to perform every conceivable test or examination at short notice and without warning.

A well-appointed bacteriologic laboratory has greater functions than mere routine diagnostic procedures. It has long been evident that selective and specific therapy should be guided by the laboratory findings and directed in consultation with the bacteriologist. At the present time, with selective antibiotic agents and sulfonamide drugs, and with the diverse possibilities of the development of resistant strains in the course of treatment, the importance of the laboratory is greatly increased. It has become necessary to determine the susceptibility of strains to the various available therapeutic substances and select the most effective. It is desirable to know that adequate levels have been maintained in the

patient and to test the effect on the bacteria concerned. If anything, the contribution of the laboratory is as important after the diagnosis is made as before. In addition, there is increasingly more reason to support the contention that the pathology of infectious processes is directly the concern of the bacteriologist.

To secure the effective performance of these functions of the laboratory, and a progressive increase in the knowledge of infections and their management, it is essential to promote the closest possible co-operation between the laboratory and the ward. Consultation, as between equals, should be the rule and not the exception. There is as much necessity for the bacteriologist to have a good knowledge of the clinical problems and difficulties as for the clinician to appreciate the possibilities and limitations of the laboratory, with purpose to benefit the patient and to advance knowledge by the co-ordination of laboratory findings and clinical observation.

Co-operation between laboratory and ward is not confined to senior members of staff. It involves those concerned with the collection of specimens and their delivery to the laboratory and no less those who supply the information upon which the laboratory worker judges what procedure and technic will give the most useful result. It involves the supply by the laboratory of adequate equipment for the collection of various suitable specimens and a statement of the information required and a willing guidance in special cases. Thus, it evidently depends upon the heads of laboratories and clinical services to promote co-operation and to organize procedure in the light of mutual understanding, good fellowship and scientific curiosity.

#### GENERAL CONSIDERATIONS ON THE COLLECTION OF SPECIMENS

The first step in the isolation and identification of pathogenic bacteria is the collec-

tion of specimens to be examined. This fundamental step often does not get the attention to detail it requires even though the results and usefulness of bacteriologic studies are dependent as much on the proper time and method of collecting the specimens as on their proper handling in the laboratory. The laboratory report can only state what is found in the specimen submitted. Failure to isolate the causative organisms of an infectious process is often due to improperly taken specimens or to lack of information on the clinical condition for guidance in the selection of suitable media and technic, or of other methods necessary for arriving at a diagnosis. Too often in a busy ward the taking of specimens is left to persons who have no proper understanding of the requirements and no knowledge of the responsibility and consequences.

Material should be collected with proper precautions from active lesions or situations where the suspected organisms are most likely to be found. It is often necessary to select particular portions of available material (such as dysentery stools, sputum, actinomycotic pus, etc.), and whenever possible such selection should be left to the laboratory. At times, the patient must contribute in the collection of a specimen and then should be fully instructed and encouraged by being given a reason. Specimens should be amply sufficient to allow of every necessary examination, particularly if several are asked for, and placed in sterile containers to avoid misleading or hampering contaminations. Once collected, the specimen should reach the laboratory as soon as possible to insure its being in a good state, and this is of particular importance when the organisms concerned are liable to injury by drying, by exposure to air and by prolonged chilling. Sometimes specimens are unavoidably small in amount, and these should not only be transmitted to the laboratory quickly but should be collected in special containers. At times it is



necessary to take the media to the bedside or the patient to the laboratory (*Neisseria*, *Treponema*, etc.) and some specimens are best collected by the bacteriologist (blood cultures). For organisms requiring unusual conditions arrangements should be made beforehand with the laboratory.

An important source of danger to the laboratory worker, to the porter carrying the specimen to the laboratory, to nurses or to other patients is the soiling of the outside of specimen containers. This receives all too little attention as a rule and can be serious. At times the entire specimen can be lost in a cotton plug (ureteral urine), or, if there is delay in delivery, it may be contaminated through a wetted plug. In any case, it is deplorable technic and indicates a lack of instruction and supervision by those in responsible positions. The danger of spread of infection by imperfectly trained help is frequently overlooked, and unwarranted reliance in them commonly relates to quite important procedures despite legal restrictions on less important matters. This applies most frequently to the collection of specimens from male patients.

Wherever possible, specimens should be taken before any treatment with sulfonamides or antibiotics is started or before wounds are treated with antiseptics. If this is unavoidable, the specimen label should tell what it may contain in order that suitable precautions be taken by the laboratory. This is becoming increasingly important.

One other important factor to be considered is the stage of the disease. The clinician should take into account which type of specimen is most likely to yield positive results during a particular phase of disease and realize when this selection is not made that negative findings do not exclude the correctness of the diagnosis; a good example is afforded by stool, blood and urine in typhoid fever. It is also a common mistake to submit samples of serum for agglutination reactions at a time when antibody response cannot be expected; much time and

energy can be expended looking for leptospira at an entirely unsuitable stage of infectious jaundice.

#### CONTAINERS AND APPLIANCES FOR THE COLLECTION OF SPECIMENS

Specimens are collected by the bacteriologist with the ordinary wire loops, pipettes, syringes, etc., the methods adopted being determined by the accessibility of the lesion, the examination to be made and the experience of the operator. The containers will be selected to be most suitable and convenient to the technic employed and to the specimen to be collected. There are occasions when specimens from particular or difficult sites are best collected by specialists in other fields, otolaryngologists, urologists, surgeons, gynecologists, etc., who will of necessity select the most suitable instruments for their purpose. The containers then used may be the routine supply or may be attachments of the special instruments used.

For the routine collection of specimens in an average hospital six forms of container meet the majority of purposes.

1. A tube or vial with a formed cotton plug in which many fluids and tissues can be put conveniently. A shell vial 25 mm. by 100 mm. long is perhaps most convenient.

2. A glass pipette 150 mm. long by 4 mm. in diameter drawn to a blunt strong point with an opening of 2 mm. in diameter. This is wrapped with cotton about two-thirds of the way from its tip to form a plug around it which fits snugly in a shell vial and holds the pipette which is also plugged. A rubber nipple allows of fluids and pus to be drawn up into the pipette and retained there when it is returned to the vial, or the fluids may be transferred with the pipette to the vial in larger quantity.

3. A finer pointed pipette wrapped and plugged in the same way as 2 but dipping into a small shell vial 30 mm. x 5 mm. within the larger one. This is for collecting small amounts of fluid, such as from the conjunctiva, chancres, tooth sockets, small furuncles and sinuses, etc. The point dipping into the small

vial prevents evaporation before reaching the laboratory.

4. A round-bottom, strong-walled centrifuge tube to fit the available centrifuges and holding 15 cc. of fluid, for blood for Wassermann and Kahn tests, for agglutination tests, etc. This saves a lot of transferring and greatly diminishes the amount of glassware to be washed in a large service.

5. Cartons of convenient size made of thick paper board and waterproofed with plastics. These sterilize perfectly in the autoclave and are most economical and convenient for sputum, feces, larger pieces of tissues, etc.

6. Cotton swabs on applicator sticks which are wrapped in cotton near the hand end to fit snugly as a plug in a 150 mm. long culture tube.

All these articles can be wrapped and sterilized in the autoclave so that they can be handled by the surgeon with his sterile gloves during an operation. As ordinarily set up they serve almost all requirements for the ordinary collection of specimens. When available, No. 2 is most used, and, once its various uses are known, it is greatly appreciated and it provides excellent specimens for the laboratory. Numbers 1, 4 and 5 have their particular uses.

The use of the cotton swab is to be avoided and discouraged as much as possible. The specimen it collects is too small and is usually dried up when it reaches the laboratory. Leukocytes and much desired material cannot be liberated again from the interstices of the cotton, and accordingly much useful information is missed because it is not only difficult but unsatisfactory to make cultures and smears from such swabs. There are sites which can only be reached with swabs, and, as the latter are considered convenient by some clinicians, they cannot be avoided entirely. A modification, formed on a length of spring wire and running in a tube which is curved so as to pass behind the soft palate, where the swab can then be extruded, used and withdrawn back into the tube, is known as a West's swab. This is used for swabbing the nasopharynx in search of meningococcus in order to avoid contamination with saliva.

Glass syringes of all sizes with the needle attached can be placed in glass tubes, stoppered with a formed cotton plug and sterilized in the autoclave. The needle is protected by a small guard tube of suitable length. Maintained in this way they are always ready for immediate use, and a lot of time and worry saved. The incidence of contaminated blood

cultures is greatly reduced if they are done with the laboratories' own syringes put up this way. This method is also equally advantageous for inoculations of man and of animals.

Public Health laboratories and those performing an outside service supply standard equipment with instructions for collecting specimens. Many specimens are received in all laboratories in pickle jars, cosmetic pots, etc., and as long as these have a good closure and the whole has been thoroughly boiled in water for not less than 15 minutes they serve the purpose. Of course postal regulations have to be observed. Laboratories can provide culture media in suitable containers for inoculation with material collected by the physician and return to the laboratory. Tubes of Loeffler serum are commonly available for the diagnosis of diphtheria and blood-collecting tubes are in daily use for the serodiagnosis of syphilis; suitable outfits for blood cultures and other purposes can easily be made up.

#### THE IMPORTANCE OF THE NATURAL REGIONAL BACTERIA IN THE EVALUATION OF FINDINGS

In order to evaluate cultural findings, it is of considerable importance to have a clear knowledge of the bacteria which are commonly found regionally in the normal human body. In this connection, it may be pointed out that the description of organisms as saprophytic and parasitic is insufficient as far as infection of the human body is concerned, since parasites like *Staphylococcus epidermidis* are nonpathogenic, while nonparasitic organisms like *Clostridium botulinum* and many other clostridia are pathogenic. Organisms which are commonly found in normal healthy persons, like the green Streptococci of the mouth and pharynx (*Streptococcus salivarius*), can under certain conditions invade the blood stream, while, on the other hand, pneumococci, Streptococci, *Hemophilus influenzae*, etc. are not infrequently found in healthy persons. Although the type of so called normal regional flora is dependent on many factors and will therefore vary quite considerably



with the age of the individual, the diet and environment, it is not easily eliminated even with disinfectants and never permanently. Casually acquired organisms, however, are fairly easily removed. Relatively little attention has been given to groups of bacteria which seem almost ubiquitous and are chiefly known to the medical bacteriologist as causes of contamination.

The normal flora of the skin at any given time will depend to some extent on the individual cleanliness and the environment. Certain organisms however seem to be present fairly consistently: for example, Welsh's *Staphylococcus epidermidis* and one which may pass for *Corynebacterium acnes*. Occasionally *Staphylococcus pyogenes*, various coliforms, proteus bacilli and other organisms are found which may cause great trouble in the interpretation of cultures from skin lesions.

Surprisingly few organisms are found in nasal cultures from healthy persons. *Staphylococcus epidermidis* and "diphtheroids" are most frequently isolated. Cultures from the paranasal sinuses in health are usually sterile. The flora of the mouth and pharynx is much more varied. The basic flora consists of *Streptococcus*, mainly of the *viridans* group, *Neisseria*, "diphtheroids" and *Lactobacillus* and a number of undefined kinds which, because they do not easily grow under ordinary circumstances, do not interfere with cultures of pathogens. In addition to these, many perfectly healthy persons harbor potential pathogens such as pneumococci, hemolytic streptococci, influenza bacilli, meningococci and still more importantly *Corynebacterium diphtheriae*. This is an important point because those people represent a constant reservoir of these pathogens (carriers). The frequency with which fusiforms and spirochaetes will be found on smears or by dark field examination depends on the condition of the teeth and gums, but there are always some of different species. The mouth and throat seem to be the natural habitat of *Actinomyces*

*israeli*, which, under certain conditions, gives rise to actinomycosis (Rosebury, 1944).

The alimentary tract flora varies in the different parts of the intestine. The empty stomach is usually almost sterile, but after meals the flora of the stomach can vary greatly, consisting mainly of bacteria which have been ingested with the food. In the normal stomach these organisms are usually rapidly destroyed by the gastric juices. In certain diseases of the stomach this self-sterilizing effect may be lost, and organisms may multiply actively; intestinal forms, especially anaerobes, may be abundant. Acid-fast bacilli, both saprophytic and pathogenic types, survive in the stomach, and gastric washings are frequently a good source for the detection of tubercle bacilli, particularly in people who swallow sputum and in infants and children whose sputum is not obtainable. The upper part of the small intestine contains very few bacteria in healthy persons. Fecal streptococci and nonpyogenic staphylococci are normally the only organisms found. The flora of the lower parts of the small intestine and particularly of the colon is quite varied; the chief forms found are colon-aerogenes species, fecal streptococci, yeasts and sarcinae, clostridia and bacteroides (Rettger and Weiss, 1937). Other little known forms can be found by careful examination.

The normal flora of the urethra consists of occasional nonpathogenic staphylococci and diphtheroids. *Mycobacterium smegmatis* which occurs in the vulva of the female and preputium of the male is of importance because it may be mistaken for *Mycobacterium tuberculosis*. The flora of the vulva consists of various organisms of the surrounding skin and rectum. The flora of the normal vagina differs before puberty and after the menopause from that of the child-bearing period, when *Lactobacillus* predominates (Weinstein, 1938). Blood, cerebrospinal fluid, urine and bile and the tissues of the body are bacteriologically sterile in health.

## MATERIALS AND METHODS

## MEDIA

The preparation and choice of culture media for the growth of pathogenic bacteria is still largely empirical, and it is only of recent years that the essential nutritional and functional needs of different species have been accurately studied (page 37). The first requirement of a medium is to grow the many various bacteria which it is necessary to isolate and identify. In order to obtain cultures easily it is desired to obtain growth of isolated colonies on a surface, making use of the propensity of bacteria to form characteristic colonies from a single cell. Media of varied formulae are used for purpose of recognition and differentiation, indicated by the evident changes which bacterial growth produces in the medium (liquefaction, hemolysis or greening of red blood cells, coagulation, acid or alkali, etc.) and by the detection of by-products of metabolism (acid, gas, indol, etc.) or of products which may be characteristic excretions or secretions of the bacteria (toxins, pigments, etc.). For this same purpose use is made of the strict requirements of certain kinds of bacteria by observing whether they will or will not grow in the presence or absence of known substances (vitamins, dyes, blood, etc.) as well as their tolerance of a defined range of acidity or alkalinity.

Up to now, purely synthetic media of completely known composition have relatively few applications in medical bacteriology. The basis of most media is an infusion of meat to which is added "peptone" (chiefly a mixture of amino acids and polypeptides), salts and sugars in known quantity. To this base agar is added to give a solid surface or form a jelly of desired consistency. A great advantage of agar is that it melts at 100° C. and sets at about 45° C., a temperature which allows the mixing of heat labile substances and of living bacteria

without fear of inactivation. Agar remains a firm jelly at required incubation temperatures. It is probable, moreover, that there is more to the use of agar in culture media than just as a solidifying agent (Hitchens, 1921). Gelatin is added for the same purpose and also to detect proteolytic activity, but is limited in use because it liquefies at 37° C., a temperature essential for the growth of many pathogenic forms. Other materials are often added to media to promote growth of fastidious species (blood, serum, ascitic fluid, pieces of fresh tissue) or to inhibit selectively the growth of unwanted species while allowing growth of others (brilliant green, tetrathionate sodium sulfite, etc.). Reducing substances are added to produce an anaerobic environment of low oxidation-reduction potential (sodium thioglycollate, cysteine, etc.). Yet other substances known to be tolerated or not tolerated or altered by organisms being studied are added for differentiation purposes.

Natural substances are often used, either in their original state or variously modified. With the exception of a few materials such as body fluids and tissues, which can be collected with strict sterile precautions, most of these undergo some change by the necessary process of sterilization or necessary adjustment of reaction to a tolerable range. Materials used in this way are minced muscle, serum, and eggs (usually coagulated by heat), slices of potato, carrot or artichoke, milk, blood and pathologic transudates into serous cavities, etc., either because they are the best known medium, show changes used as diagnostic criteria, or promote particular characters of selected organisms.

Special differential media are made by supplying the bare growth requirements of specified kinds of organisms and adding pure substances, the use or not of which it is desired to know. The base must, of course, be free of the substance to be tested or of substances which would cause



confusion. The commonest substances used in this way are the so-called "sugars," such as glucose, lactose, mannitol, etc.; their utilization by bacteria is indicated by the production of acid as revealed by changes in a pH indicator, or by the production of gas fracturing the medium or accumulating in an inverted tube. This principle is also used for detecting the selective utilization of a "sugar" by organisms in a separate single colony on the surface of an agar medium. In this case, one observes changes occurring round the colony in an indicator added to the medium. Thus, acid production from lactose by a colony can be readily detected and permits the selection of a particular colony from among many which otherwise look alike. This is an important practical application.

In order to make use of these functional applications of culture media it is imperative that only the bacteria present in the material to be studied be allowed to grow. It is therefore necessary that the medium be collected completely free of bacteria or rendered absolutely sterile and inoculated with proper technic only with the material to be tested. The most convenient and certain method of sterilization is steam under pressure in the autoclave and this is used wherever heat and moisture are not injurious to the material. Where heating under pressure is undesirable, sterilization can be obtained by the process of tyndallization (steaming on 3 consecutive days) in an Arnold sterilizer. For still more heat labile materials, micro-organisms can be removed by filtration through diatomaceous earth (Berkefeld type), porcelain (Chamberland type) or sintered glass filters.

This is not the place to give a formulary of the hundreds of different media that have been described. Mention of certain ones will be made as examples, but for fuller information reference should be made to books and papers devoted to media and their mode of preparation and sterilization.

#### TEMPERATURE AND TIME OF INCUBATION

In addition to food requirements, there are other important factors to be considered to allow for optimum bacterial growth; one of them is temperature. Most pathogenic bacteria grow best at about 37° C. and the temperature has to be maintained uniformly for a suitable time. Incubators at 10° C., 20° C. and 37° C., as well as means of providing higher and lower temperatures, should be available because temperature tolerance provides differentiating criteria for certain species. Some bacteria have a relatively wide range of tolerance while others grow only within very narrow limits. In any case most rapid and profuse growth usually demand a fairly definite optimum range; above and below it growth is slowed and reduced. An improper temperature cannot be compensated for by increased time of incubation. The majority of pathogenic bacteria develop visible colonies after 18 to 24 hours of incubation at optimal temperature, but there are some very notable exceptions which have to be considered to avoid missing important organisms. *Mycobacterium tuberculosis* requires at least several days even on the most favorable media and may require weeks before growth is obtained. In most laboratories cultures of suspected tuberculous material will be kept from 6 to 8 weeks before being discarded as negative. Promise of quicker results is indicated by the reports of Foley (1946) and Goldie (1947), using a medium described by Dubos et al. (1947). It is also recommended to keep cultures for *Brucella abortus* an average of 4 weeks before a negative report is made. Between the fast-growing organisms and the ones that require weeks for development there are many exhibiting a wide range of requirements of incubation time. This is often characteristic either for the production of a certain reaction or for the development growth. Thus, there is a definite time and a definite temperature which are best for the production of diph-

theria toxin, and these differ from those required for the production of tetanus toxin or staphylococcus toxin. These considerations apply to the primary isolation of organisms and even more so to the various characteristic reactions in differential media.

#### ATMOSPHERE

The general principles dealing with the effect of oxygen on bacterial growth have been discussed (pages 27 and 355), and only the practical applications will be emphasized here. It is a sound rule to set up all cultures in duplicate and to incubate one set aerobically and the other anaerobically whenever conditions permit. The extra amount of labor involved will be repaid richly by more reliable results with clinical material. The role of certain anaerobic or microaerophilic organisms in disease processes is still obscure in many instances, and only systematic studies will give adequate realization of their importance.

Various methods are available for the production of anaerobic conditions. Brewer's (1940) thioglycollate medium permits the growth of anaerobes under the usual technic of incubation used for aerobes and can therefore be used by small laboratories which have no anaerobic equipment. As it does not provide adequately for the growth of surface colonies, isolation of pure cultures is only possible through shake cultures and the picking of colonies in the body of the medium. However, the thioglycollate medium is excellent for subcultures and for the examination of many special characters of anaerobic cultures. Other media containing reducing substances have been recommended for the growth of anaerobes under routine conditions of incubation. Thus, glucose has long been known to create partial anaerobic conditions by its reducing action. The addition of a small amount of agar (0.1 per cent to 0.3 per cent) produces slightly viscid media which provide various degrees of anaerobiosis (Falk et al., 1939). Cysteine (0.3 per cent) added to agar or infusion broth acts as a reducing agent. These methods also present the same disadvantage with mixed cultures and necessitate replating in order to permit isolation of pure cultures.

Special culture dishes have been designed in an attempt to avoid this trouble (Brewer, 1942; Spray, 1931 and 1936). Another simple device which has the same disadvantages is the deep inoculation into media freshly boiled to drive out dissolved gases, and attempts at physical exclusion of oxygen by pouring liquid sterile paraffin or petrolatum on top of the inoculated medium.

Absorption of oxygen by chemicals does not require any special apparatus. Any jar with a well-fitting lid can be used. An alkaline solution of pyrogallol absorbs large quantities of free oxygen. A dish containing pyrogallic acid is put in the bottom of the jar. The culture plates or tubes are put on a wire rack above the dish. A 5 or 10 per cent sodium-hydrate solution is added to the pyrogallic acid, and the jar is quickly sealed with a suitable lute. The absorption of  $\text{CO}_2$  by the alkali may at times interfere with the growth of organisms requiring it. Similarly cultures of various bacteria which consume oxygen rapidly or a burning candle can be added to sealed jars to remove oxygen from the atmosphere.

Specially constructed jars for the cultivation of anaerobes are based on the combination of oxygen with hydrogen by catalytic action. The McIntosh and Fildes (1921) jar uses palladinized asbestos as catalyst in a small wire cage surrounding a small electric bulb which provides heat to start the reaction. The jars are made either of metal or glass and the lids have to be well fitted and provided with terminals to the heating element and with stopcocks to introduce the hydrogen; the pressure is controlled by means of a manometer. These jars permit the growth of all types of cultures, and pure culture isolation from surface colonies is as easy as with aerobes. They afford complete anaerobiosis which is essential for some organisms. Various modifications of the same general principles are in use. The modification by Brewer and Brown (1938) and Brewer (1939) permit the use of illuminating gas. Chromium sulfuric acid mixture in the bottom of an acid resistant jar has been recommended by Rosenthal (1937). The method is cheap and simple: 15 per cent solution of sulfuric acid (100 cc. per litre capacity) and 5 grams of chromium metal (per litre) are put in the bottom of a jar. The plates and tubes supported on a stand are put in and the jar is sealed. The lid must contain a gas outlet which is closed after the vigorous formation of hydrogen has subsided. Whatever method is used a tube of dex-



trose broth containing a small amount of methylene blue should be placed in the jar to test anaerobiasis by the reduction of the dye.

Increased concentrations of carbon dioxide is required by certain organisms especially on first isolation, e.g., *Brucella abortus*, *Neisseria gonorrhoeae*. *Staphylococcus pyogenes* produces its specific toxin in much higher quantities in an increased CO<sub>2</sub> tension.

Any of the jars used for anaerobic cultivation can be used for the carbon dioxide method. The desired amount of air is evacuated and replaced by CO<sub>2</sub> from a tank; an atmosphere containing 10 to 18 per cent CO<sub>2</sub> is usually required. Another method to obtain increased CO<sub>2</sub> tension is the following: place in an open container 0.24 Gm. of sodium carbonate and 4 cc. of 10 per cent sulfuric acid for each litre capacity of the jar used; when the chemical reaction begins to subside seal down the lid and incubate. Special methods have been devised to increase the CO<sub>2</sub> tension inside individual culture bottles (Huddleson, 1939; Shaughnessy, 1939).

#### TECHNIC OF INOCULATION AND SUBCULTURE

The working bench in any bacteriologic laboratory should be kept scrupulously clean; this will decrease the occurrence of accidental contamination of cultures. A flame, preferably a Bunsen burner with pilot light, is indispensable. A jar of lysol or any other suitable disinfectant should be provided for the disposal of contaminated pipettes, etc. A stand for the various loops and needles is another necessary item. Stiff platinum irridium wire or nichrome wire in glass or metal handles is generally used to transfer cultures; the number and types of straight wires and loops depend on individual taste. Every laboratory worker has a preferred set which fits his own needs. One straight wire for stab culturing and picking off colonies, two loops of different size and one bent wire to use as a spreader will be sufficient for almost any kind of work. Sterile forceps and scissors will be found useful on many occasions, as will a sterile pestle and mortar. Glass tubing (Pasteur pipettes, Wright pipettes) with one end drawn out to a fine point, the other plugged with cotton wool, to which a rubber nipple can be attached, prove very helpful on many occasions. The flaming

of all tools before and immediately after use must become an automatic action, and care must be taken to flame not only the tips of wires and loops, but also the part of the handle which will be inside the tubes when inoculating.

The transfer of material from their sterile containers to the culture media requires a certain experience in order to judge the optimum size of inoculum and how to spread it to obtain well-separated discrete colonies. The size of inoculum used depends to a certain extent on the number of bacteria expected in the particular kind of specimen; some assistance may be derived from examining a stained smear. When fluid material is cultured it is often necessary to centrifuge the specimen and use the sediment as inoculum; on the other hand, if a specimen is likely to yield a very heavy mixed flora it may be of great advantage to streak two or even three plates in succession without going back to the original material or flaming the spreader between one and another. By this method it is often possible to get well-isolated colonies on the second or third plate. It should be made a general rule to study Gram-stained films carefully, because this will facilitate and direct the choice of culture media.

Plates and culture tubes are held at a slight angle and in such a way that the worker can see the inoculating needle. Plates are incubated in an inverted position to prevent the water of condensation which will collect on the lid from dropping onto the inoculated plates. Tubes are incubated in an upright position in suitable racks. Correct labelling of all cultures is a very important detail, because any error of this type may have disastrous results.

The reading of plates after incubation and the picking of colonies requires a certain skill and some experience and guidance. The purpose of picking single colonies is to get pure cultures for further identification; it is obvious that only if the resulting growth is pure will further tests be reliable. The plates should be read under a good lens which permits close inspection, and colonies should be picked with a straight wire. The picked colony is trans-

planted to a slant by even stroking over the surface, or into a fluid medium by emulsifying against the wall of the glass.

Tubes are usually plugged with cotton or, even better, with screw caps. The plugs are removed with the little finger, or between third and fourth finger. The mouth of the tube is flamed before inoculation to burn away shreds of fibre from the plug, and again before the plug is put back. Care should be taken to avoid touching the mouth or sides of the tubes with the inoculating needle. After renewed incubation, the purity of cultures should be checked by Gram smears before any further tests are done. If the cultures are mixed either another colony has to be picked from the original plate or the mixed culture must be replated.

Original plates should always be kept until all desired colonies have been picked and have grown on transfer. Another reason for keeping the original plates is that certain types of organisms grow slowly and require prolonged incubation. This is particularly the case with some anaerobic organisms. Actinomycetes, for instance, will be missed unless plates are incubated for at least 5 to 7 days. Cultures from sputum, stools, and other materials which always give a very mixed growth present quite a problem to the beginner. It will be of great advantage whenever time permits to smear and transfer all the various types of colonies until the worker becomes fairly familiar with colony form and is able to recognize those characteristically belonging to the "normal flora" of the kind of specimen. In examining a primary culture not only the absolute number of colonies should be noted, but, in mixed cultures, it is of importance to observe the relative numbers of the different types present. The absence of organisms which one normally expects to find in any kind of specimen may have clinical significance and should be noted down and reported.

Accurate record keeping is an absolute necessity. The description of colony form, relative numbers, staining reaction and morphology as well as the various steps for iden-

tification should be clearly written into the records, with dates of subculture and results. These details may seem rather time consuming, but only if these rules are strictly followed can the record be referred to again at some later date on the return of a patient, or use be made of the wealth of material usually collected in a laboratory. For laboratory use, the final conclusions and reports to the clinician can be summarized briefly and to the point, so long as it gives an accurate impression.

### PRESERVATION OF CULTURES

Several methods can be used to preserve cultures for further work, for teaching purposes, etc. The simplest is sealing the slants or tubes in which the pure culture has been grown. Meat-mash media will keep many cultures viable for some time. The safest way of preserving a culture for indefinite periods consists in drying it in vacuo from the frozen state or in drying small pieces of filter paper impregnated with a drop of culture (Swift, 1937). The addition of vitamin C is claimed to increase the viability of dried cultures (Stamp, 1947).

Inspissated egg medium is useful for preserving cultures; after incubation for 18 to 24 hours the cultures should be kept in the ice chest (4° to 6° C.) and transfers made from time to time. This method is useful if living cultures are necessary fairly frequently for some tests, such as agglutination, opsonocytaphagic index of *Brucella*, etc. A laboratory collection of reliable cultures is essential and must be very carefully maintained, not only to keep the cultures alive but to be sure that the labels are correct. The collection must be in the charge of a senior and responsible person.

### EXAMINATION OF MATERIAL FROM PATIENTS

#### BLOOD CULTURES

The isolation and identification of bacteria from the blood stream is often of considerable importance, and much information concerning the pathogenesis of diseases has been gained by this means. Quite fre-



quently the invasion of the blood stream by bacteria is the first clinical manifestation of some local lesion and the type of organisms isolated may draw the clinician's attention to the possible site of the focus. However, it is very important to realize that a bacteremia is very often transitory, and unless the blood culture is done at the appropriate time it may not be successful. In a patient with typical clinical signs and symptoms of a septicemia, there is usually no great difficulty in the isolation of the causative organism, but in cases of pneumonia, typhoid fever, meningococcus meningitis, etc., the respective bacteria may be found in the blood during certain stages only. In the first week of typhoid fever *Salmonella typhosa* will be found in the blood with great regularity in 89 per cent of cases (Callman and Buxton, 1907), during the second week in 73 per cent, during the third week in 60 per cent, during the fourth week in 38 per cent, and in the fifth week in 26 per cent. Meningococci can be isolated from the blood in the very early stages of the disease, and blood cultures are particularly valuable for diagnosis in cases without meningeal symptoms. Blood cultures may be of great help in the treatment and prognosis of a severe pneumonia, although the use of specific antiserum in the treatment of pneumococcus bacteremia and lobar pneumonia has been almost completely abandoned since the introduction of the sulfonamides and penicillin. Bacteremia is very often present without causing the expected clinical picture commonly associated with it (high fever, prostration, etc.). Brucellosis and bacterial endocarditis due to *Streptococcus salivarius* (*viridans*) in many instances begin insidiously with very little elevation in temperature or any prominent signs of bacteremia, but cultures of the blood may establish the diagnosis and provide a basis for rational treatment. On the other hand, neither single nor even multiple negative cultures are definite evidence against the diagnosis established on other grounds in

diseases which usually show a septicemia.

Since the widespread use of sulfonamides and more recently of penicillin and streptomycin, the numbers of positive blood cultures have been decreasing steadily among hospital patients. Treatment with these drugs often starts at home at the onset of a febrile disease and is continued if the patient is sufficiently ill to be admitted to a hospital. In consequence, the correct diagnosis cannot be made in many instances. Unless not only the bacteremia but the focus which caused it has been eradicated, a second blood stream invasion may occur, and then the increased resistance of the organisms to the various drugs may prevent effective treatment.

**Technic of Blood Cultures and Necessary Equipment.** Two well-fitting, sterile, dry, 30 cc. syringes with 18 or 20 gauge needle set up in sterile glass barrels are required, for if the first puncture should be unsuccessful the second syringe is immediately ready, thus eliminating the risk of clotting and of contamination due to changing needles; sterile gauze swabs; 5 per cent watery solution of iodine in 10 per cent potassium iodide; 70 per cent alcohol; alcohol lamp; culture media as indicated for the specific case; tourniquet.

The skin over the median vein in the antecubital fossa is cleaned with alcohol, then painted with iodine and washed once more with alcohol. The desired quantity of blood is drawn, the tourniquet is loosened, the syringe withdrawn, a sterile gauze sponge pressed on the point of puncture, and the arm is raised without flexure at the elbow. The blood from the syringe is immediately distributed into the culture media with bacteriologic technic. The antecubital fossa is the site of choice for vein puncture, but other sites have to be used occasionally. In babies and infants it is often necessary to use the external jugular vein as site of puncture; this procedure should be done by an operator experienced in the technic.

The clinician should notify the laboratory

if the patient has received sulfa drugs or antibiotics before the blood culture was taken. The addition of 1 mg. of para-aminobenzoic acid to 100 cc. of medium is sufficient to antagonize the bacteriostatic action of the ordinary levels of sulfonamides, but very high sulfonamide levels may make it necessary to use 5 mg. per 100 cc. (Janeway, 1941). To antagonize penicillin, clarase or penicillinase (Lawrence, 1943 and 1945; Woodruff et al., 1945) should be added to the media and the blood taken, if possible, just before the next dose, when the level in the blood is lowest. Streptomycin can be inhibited by the addition of from 3 to 5 mg. of cysteine per 100  $\mu$ g. of antibiotic (Denkelwater et al., 1945; Geiger et al., 1946).

As previously mentioned, the culture taken at the bedside by experienced technicians will give the best results. However, this is not always possible, and it may be necessary to ship blood over some distance to a laboratory (postal regulations). It must be clearly understood that under such conditions results will depend upon the organism present in the blood, the time lapse between withdrawal of blood and its addition to media and incubation, and the varying temperatures to which the sample is exposed during transport. Generally speaking, the safe temperature range for pathogenic bacteria is between a minimum of from 15° to 20° C. and a maximum of 43° C. (Tanner, 1928). Certain bacteria like meningococcus, gonococcus, pneumococcus are highly susceptible to rapid changes in temperature. If blood is shipped to the laboratory, sodium citrate or heparin has to be added to prevent clotting, or it must be shipped in proper containers containing medium. Successful isolation has been made from clotted blood sent to the laboratory in the ordinary way for Widal reactions (Martin, 1931 and 1932).

The amount of blood withdrawn and the media used vary widely in different laboratories. It is important that some indication be given by the clinician concerning the

type of case, because this will determine the choice of media, the conditions of incubation and the method of examination. For most organisms, beef infusion broth and nutrient agar are sufficient. In the McGill University laboratories 2 flasks of 190 cc. of beef infusion broth and a large tube of 76 cc. agar are used routinely; 10 cc. of blood are added to each of the flasks and 4 cc. of blood to the agar at 45° to 50° C., from which 4 plates are poured. One flask and 2 plates are incubated anaerobically, and 1 flask and 2 plates aerobically at 37° C. After 18 to 24 hours' incubation, the plates are inspected for deep colonies and the flasks for turbidity and for colonies on the surface of the sedimented cells. After shaking, hanging drops and smears are made from the flasks, and a large drop is inoculated onto suitable plates. This procedure permits the growth of staphylococci, streptococci, pneumococci, meningococci and of organisms of the coli-typhoid group, of influenza bacilli as well as of many other organisms occasionally isolated from subacute bacterial endocarditis. Most clostridia, anaerobic streptococci, and bacteroides will also grow in the anaerobic cultures of this type. If the cultures are negative after 24 hours, they should be incubated further, inspected daily, and daily subcultures made from the fluid medium. Certain organisms are slow growing, particularly some types of *Streptococcus viridans* of subacute bacterial endocarditis which often require 10 to 15 days' incubation. Once growth is obtained, the identification of the organism is based on a variety of criteria; morphology, staining reactions, cultural reactions, resistance, metabolism, fermentation reactions and other biochemical properties, antigenic structure and pathogenicity.

Cultivation and isolation of certain organisms from the blood stream are best obtained with special enriched culture media. The isolation of *Brucella* is rather difficult and various methods have been described for it (Poston, 1941; Huddleson, 1939;



Wise, 1943). *Brucella abortus* often requires 10 per cent CO<sub>2</sub> for primary cultivation; growth may be very slow, and cultures should not be discarded for 4 weeks. The isolation of *Brucella suis* or *Brucella melitensis* from the blood stream is usually less difficult, and growth will occur under aerobic conditions. However, isolation of *Brucella melitensis* often requires that the culture be taken at the evening rise of temperature and at the height of a bout of fever (Zammit, 1905; Shaw, 1905; Eyre et al., 1907). For purposes of identification and differentiation of one species from the other, all available methods should be used: requirements with reference to CO<sub>2</sub>, growth in the presence of dyes, H<sub>2</sub>S formation, agglutinin absorption tests and virulence. Bacteremia and septicemia due to *Neisseria gonorrhoeae* are rare but if they occur they are usually accompanied by widespread infection throughout the body with abscesses, endocarditis, etc. Growth of the organisms from the blood is facilitated by incubation in moist jars in which from 8 to 10 per cent of the air has been replaced by CO<sub>2</sub>.

Two types of organisms seem responsible for rat-bite fever in man. *Streptobacillus moniliformis* can be cultured from the blood during a febrile attack. Twenty cubic centimeters of blood are distributed in serum broth and streaked on Loeffler's serum plates (Topley and Wilson, 1938). Incubation can be aerobical or anaerobical, preferably in the presence of 5 per cent CO<sub>2</sub>. The second organism responsible for rat-bite fever is *Spirillum minus*; the bacteriologic diagnosis can be made by subcutaneous inoculation of mice or guinea pigs with blood taken at the height of the febrile bout. The blood of the animals should be examined daily from the fifth to twentieth day under the darkfield microscope or after staining with ordinary dyes.

It has been stated by Simpson (1929) that an early bacteremia occurs in tularemia in man and that blood cultures taken between the third and tenth day of disease

may be positive. Successful isolation of *Pasteurella tularensis* from the blood on blood dextrose cystine agar has been reported by Rausmeier and Schaub (1941). More commonly, 5 to 10 cc. of defibrinated blood is injected intraperitoneally into guinea pigs. If *Past. tularensis* is present in the blood, the animals will die within 10 days; cultures should be made from the blood, lymph nodes, liver and spleen. Special precautions have to be taken by the laboratory worker in handling any material from a suspected case of tularemia and in the post-mortem examination of animals. The organism is extremely infectious for man, and the laboratory should receive very definite warning when suspected specimens are sent, a simple precaution which is neglected only too frequently.

On occasions, it may be of great practical and theoretical interest to do blood culture on obscure types of diseases where the clinical picture suggests an infectious process. In such cases it is desirable that the clinician discuss the problem fully with the laboratory worker. Negative results in routine cultures are of limited value, and several attempts under special conditions often with complex and careful methods may be necessary. The interpretation of positive findings is not always as easy as is commonly assumed. The isolation of the corresponding organism from typical clinical cases of pneumonia, typhoid fever, etc., can be taken as sufficient evidence of causal relationship. However, isolation of organisms from the blood stream of healthy people or after extractions of teeth have been reported. These findings represent in most instances transitory bacteremias and are usually due to organism of low pathogenicity.

Contaminations, often due to faulty technique but sometimes unavoidable, may complicate the interpretation of blood cultures. The percentage of contamination is a good index of the technic and should be kept to a minimum. Contaminants can usually be

recognized either by their nature or by their distribution in the various culture media, but critical judgment must be applied, and positive findings should not be disregarded by the laboratory worker or clinician because the organism isolated is usually considered nonpathogenic. Any organism isolated from suspected cases of subacute bacterial endocarditis or other obscure chronic infectious condition must be carefully studied and the blood cultures repeated. Shiling (1939, 1940) in his survey on the bacteriology of endocarditis was able to compile a list of 28 different types of bacteria; he felt that clinical, bacteriologic and pathologic evidence was sufficient to consider these types as causative organisms.

Pathogenic organisms are sometimes introduced into the blood culture from the improperly prepared site of venipuncture (infected skin lesions) or from contaminated bed clothes. If the slightest doubt exists about the etiologic relation of the isolated bacterium to the condition of the patient, the blood culture ought to be repeated immediately.

#### CEREBROSPINAL FLUID CULTURES

In any suspected case of acute or chronic meningitis the culture and examination of spinal fluid will greatly contribute to diagnosis. Spinal fluid must be collected with sterile precautions and sent to the laboratory immediately, before possibility of clotting. In cases of acute meningitis, the C.S.F. may vary from very slight turbidity to thick purulence; the cellular elements consist almost entirely of polymorphonuclear leukocytes, though in later stages mononuclear cells may be numerous. The most common organisms causing acute meningitis are meningococci, pneumococci, tubercle bacilli, less commonly influenza bacilli (much more frequent in children) and uncommonly staphylococci and many other organisms.

A Gram stain will often permit a presumptive diagnosis and the choice of appropriate culture media for the growth of the causative organism. Purulent spinal fluids in which no organisms can be found after careful search suggest meningococcal meningitis. Meningococci autolyze very quickly and therefore may not be found on smears, although the fluid may give a positive precipitin reaction with the appropriate anti-meningococcus serum. If sufficient pneumococci, meningococci, influenza or Friedländer's bacilli are found on smear, the direct serologic type identification of these organisms should be attempted.

**Pneumococcus.** Drops of turbid spinal fluid are put on a clean slide and mixed with a drop of pneumococcus type antiserum and some methylene blue and examined under the microscope for capsule swelling. The antiserum must be potent, type specific and show no cross reactions, and the amount used must be adjusted to the relative numbers of organisms present or capsular swelling may be delayed or suppressed completely. An interesting observation made by Fisher (1947) should be kept in mind: in specimens taken from patients who have been treated with penicillin, "Quellung" of the capsules was obtained although no pneumococci or only very faintly stained cells were visible within the capsule—the "empty capsule" phenomenon.

**Meningococcus.** If Gram-negative intracellular or extracellular diplococci are found on the smear of spinal fluid, a provisional diagnosis of meningococcus meningitis can be made. The examination of the smears should be made very carefully, as errors due to staining technic may occur. (Gram-positive cocci appearing Gram negative) and as strains of *H. influenzae* can be sometimes very pleomorphic and mistaken for cocci. Occasional cases are due to gonococcus and can only be identified by culture. The spinal fluid should be cultured and the organism identified by its morphologic, bio-



chemical and serologic characters. Occasionally, it is possible to make a quick reliable diagnosis directly from the C.S.F. by the capsular swelling test (Clapp et al., 1935) but this requires that sufficient organisms be present in the C.S.F.; the same technic is used as for pneumococcus typing.

**Hemophilus Influenzae.** Any C.S.F. showing Gram-negative, pleomorphic rods should be set up immediately for the quick identification of *Hemophilus influenzae* by capsule swelling, using the same technic as for pneumococcus. In this particular instance early and exact determination of the type causing the infection is very important, since type-specific antiserum is the treatment of choice and should be given as early as possible (Alexander, 1935, 1943); streptomycin has recently been used with promising results (Alexander, 1946). Six types of influenza bacilli have been recognized by Pitman (1931); practically all cases of *Hemophilus influenzae* meningitis are caused by type B, but occasional cases caused by types A and F have been reported. The organisms grow as minute colonies on blood agar and give large colonies in the vicinity of a streak of staphylococcus. They grow readily on Fildes serum agar and on Matthews medium.

**Klebsiella Pneumoniae** (Friedländer's bacillus) is rare as the causative organism of meningitis. Stained smears show Gram-negative rods surrounded by a large capsule. Typing sera, when available, allow specific diagnosis. Growth is readily obtained on ordinary media and is easily identified.

**Mycobacterium Tuberculosis.** Tuberculous meningitis is a fatal and not infrequent occurrence. The most rapid diagnosis is made by finding characteristic acid-fast rods in suitably prepared films. Because the cerebrospinal fluid clots quickly, a special manipulative technic is required to make satisfactory films. The simplest is to collect the fluid directly from the spinal tap

into small vessels into which a coverslip fits fairly snugly; let the clot form, settle and shrink; remove the exuding fluid but do not manipulate the clot in any way. The coverslip is then removed, allowed to dry, fixed and stained. Another method is to collect about 7 cc. of fluid in test tubes and allow to clot. The clot is then poured out carefully onto slides laid in petri dishes in such a way that it lies lengthways on the slides without manipulation. The clot settles on the slide, exudes fluid and shrinks onto the glass. The slides are removed, allowed to dry thoroughly, fixed and stained. In both methods great emphasis must be placed on strictly avoiding manipulation of the clot because it will contract firmly and unevenly with every touch, to such an extent that it may become useless for examination. Cultures are made on the usual media for growing *Mycobacterium tuberculosis* (Lowenstein, Petragnani, Dorsett's egg) and growth is obtained in a matter of one or two weeks. Sometimes cultures made unsuspectingly on ordinary laboratory media may yield growth in about two weeks. Guinea-pig inoculation may have to be resorted to for definite diagnosis as in other cases of tuberculosis. The fluid in tuberculous meningitis may be clear or faintly cloudy and exhibit chiefly lymphocytic cells although varying proportions of polymorphonuclear leukocytes may be present.

Brewer's thioglycollate broth and blood plates with staphylococcus streaks will permit the growth of the common pyogenic organisms causing acute meningitis. Unusual forms such as *Actinomyces* and other anaerobes are obtained in a few cases. If fungi are suspected the sediment should be plated on Sabourand's slants or other selective media as well as on the ordinary media. Sabourand's medium should be incubated at 37° C., and duplicate sets left at room temperature. Animal inoculations are sometimes effective.

EXUDATES FROM EYE, EAR, NOSE, THROAT  
AND PARANASAL SINUSES

Washings from the conjunctivae are often cultured before operation on the eye by inoculating thioglycollate medium and a blood-agar plate with a streak of *Staphylococcus* culture to aid in the detection of *Hemophilus influenzae*. The most common organism in the conjunctiva is *Corynebacterium xerosis*, a harmless saprophyte which can be ignored as can *Staphylococcus epidermidis*. The finding of any pathogenic or potentially pathogenic organisms should be immediately reported in order that treatment with sulfonamides or an antibiotic be started and the conjunctival sac sterilized. Gram-stained films of discharges from diseased eyes may give a clue as to the implicated micro-organism. Most of the common pyogenic organisms can cause conjunctivitis, *Staphylococcus pyogenes*, *Streptococcus pyogenes*, *Hemophilus influenzae* and pneumococcus, rarely meningococcus; severe forms are caused by gonococcus and *Corynebacterium diphtheriae*. Angular conjunctivitis is caused by *Moraxella lacunata* and conjunctivitis with ulceration by *Moraxella liquefaciens*, both characteristic short Gram-negative diplobacilli. These possibilities have to be kept in mind when choosing media for plating, but it is only necessary to make particular mention that (1) *Moraxella lacunata* will only grow on Loeffler inspissated serum and must be subcultured from the pits it makes in the medium, and that (2) although *Moraxella liquefaciens* also grows well on Loeffler serum, its great proteolytic activity renders isolation difficult. Fortunately, *M. liquefaciens* will grow satisfactorily on blood agar.

Discharges from ears in acute, subacute or chronic otitis media usually yield pyogenic organisms and have to be treated accordingly. *Proteus* and *Pseudomonas* are often found in discharges from chronic otitis media. The same type of flora occurs

in mastoiditis. Very rarely will *Actinomyces* or *Mycobacterium tuberculosis* be found in these specimens, but the possibility must be considered, particularly if there is any suggestion in the history.

Nasal secretions may yield any of the respiratory pathogens. In babies and infants nasal swabs have to be used instead of sputum for cultivation; they are usually a reliable index of the organisms causing the pneumonia. The incidence of *Staphylococcus pyogenes* in nasal and throat swabs in healthy persons is quite variable. As already mentioned, the paranasal sinuses are sterile in health or contain at most only a few saprophytes which may originate from contamination during the collection of specimens. In disease, the flora of the sinuses may be quite varied and contain almost any kind of bacteria. Infection of the maxillary antra may occur through apical tooth abscesses and in such cases different types of anaerobic organisms may be found. The inspection of the specimen, its description as mucoid, muco-purulent or frankly purulent, its odor, and the study of a Gram-stained film will be a guide in the choice of media. Swabs from throats or tonsils are most often taken in cases of acute tonsillitis and will yield *Streptococcus pyogenes* in the majority of cases. The proof of or exclusion of diphtheria is often requested from the laboratory and is very important because of the occasional case of modified diphtheria following an imperfect response to immunization with toxoid. These cases show no more than a fairly profuse serosanguineous exudate which is more often nasal than pharyngeal. The physician must realize that the exact laboratory diagnosis takes too long and can therefore only be confirmatory. The decision as to whether antitoxin should be used rests with the physician. If a typical membrane is sent to the laboratory and the smear shows characteristic organisms in large numbers this fact should be immediately reported to the clinician.



A negative smear of a poorly taken throat swab does not exclude the diagnosis of diphtheria, and the clinician should not be influenced in his clinical judgment by this finding.

The diagnosis of Vincent's angina can be confirmed microscopically and best by dark-field examination of fresh specimens which reveal the characteristic active movement of the organisms and their solid appearance and delicate form. The cultivation of the fusiform is hardly worth the great trouble, and *Borrelia vincenti* cannot be grown at all. Simple Gram stains or films stained by steamed gentian violet will show the characteristic picture of spirochetes and fusiforms in very large numbers. *Borrelia vincenti* cells are thin, with open coils and pointed ends and *Fusobacterium plauti-vincenti* of medium size, thin and gradually tapered; both are more delicate than related species. The diagnosis is not easy and should not be based on a few spirochetes and fusiforms, especially when they are of large size, sluggish and double contoured as seen by dark-field examination.

Large round or oval budding yeast cells (Gram positive) and occasional short thick filaments will be found in smears of thrush lesions—whitish or creamy, easily removable membranes. *Candida albicans* is the causative fungus and can be grown and identified.

#### SPUTUM AND BRONCHIAL SECRETIONS

Sputum is one of the most common specimens received in most diagnostic laboratories, especially in cases of acute upper respiratory infections, pneumonias, etc. The organisms most frequently found are pneumococci, influenza bacilli, Friedländer's bacilli, various streptococci and rarely staphylococci, in various combinations. In infants fulminant and rapidly fatal cases of tracheo-bronchial staphylococcus infection have to be differentiated from tracheal diphtheria and from occasional severe tracheitis due

to *Hemophilus influenzae*. In anaerobic cultures from some cases a number of non-spore-forming anaerobes difficult to identify and to evaluate are found. In typical cases of lobar pneumonia the sputum is "rusty" and shows on smears and cultures practically nothing but pneumococci which can be typed directly by the quellung reaction. Acute or chronic infections due to *Klebsiella pneumoniae* (Friedländer's bacillus) show a thick tenacious sputum; there is no difficulty in isolating and identifying the organism. Sputum from chronic infections of the bronchi or from bronchiectasis may show a great variety of aerobic and anaerobic microbes. It is often difficult to evaluate cultural results in these cases, particularly if the flora consists of organisms generally considered as saprophytes; of others little or nothing is known.

Cultures of sputum or, better, secretions from lung abscesses may yield a pure growth of any of the pyogenic pathogens, but more frequently mixtures of organisms; at times numerous borrelia and fusobacteria, which are difficult to identify, are found.

In obscure, usually subacute or chronic, lung lesions, sputum is often sent to the laboratory for fungus cultures. *Candida albicans*, *Coccidioides*, *Histoplasma capsulatum* or *Aspergillus* are the fungi which may cause primary lung infections. Great care must be taken in evaluating the culture findings, particularly in the case of *Candida albicans* or *Aspergillus*, both of which may occur as secondary contaminants. Only if fungi are found constantly on repeated examinations in large numbers should the probable diagnosis of fungus disease be made by the laboratory. (See Chapter 32 for details of cultivation, etc.)

All specimens of sputum or bronchial secretion should be studied in films stained by Gram stain; Wayson's stain may sometimes be helpful, and the specimens should also be inspected carefully for any particular granules or bronchial plaques. Blood

plates, streaked across with staphylococcus for its satellitic effect, and incubated aerobically and anaerobically, are usually sufficient unless special culture methods need to be used when tularemia, fungus infections or other unusual conditions are suspected.

**Pertussis.** The most suitable method for the isolation of *Hemophilus pertussis* is the "cough-plate." A Petri dish containing Bordet-Gengou medium is held 5 or 6 inches from the patient's mouth during a coughing spasm; the droplets expelled by coughing are the best inoculum obtainable, and the method gives the best colony distribution. Gardner and Leslie (1932) very strongly recommend waiting for a spontaneous coughing spell rather than inducing coughing as is sometimes done. As it is important to avoid mucus plugs and saliva, sputum and swabs are not very satisfactory. It is also important to incubate the plates as soon as possible after inoculation. Bradford and Slavin (1940) use swabs on flexible copper wire passed through the nostril to the nasopharynx to avoid saliva.

Tuberculosis provides the laboratory with the greatest number of respiratory tract specimens, the most usual being sputum, bronchial aspirations and stomach washings. As all these specimens contain organisms which would interfere with the very slow-growing *Mycobacterium tuberculosis*, various methods have been devised to destroy the contaminants before culturing the specimens. The direct microscopic examination of Ziehl-Neelsen stained films will reveal the tubercle bacilli only if they are present in fairly large numbers, so undue reliance must not be placed on making a diagnosis from a microscopic examination alone. There is no sure method of distinguishing tubercle bacilli from other types of acid-fast saprophytes on a stained film. Absolutely clean new slides must be used, as an occasional acid-fast rod can be found on previously used slides which have been cleaned; the staining reagents should be made with fresh distilled water. After read-

ing positive films, the oil immersion lens should be wiped, and care must be taken that the oil dropper does not get contaminated. Digestion or concentration of the specimens prior to making films will give more accurate results by insuring better distribution; the methods of Andrus et al. (1924) or of Hanks (1938) are recommended.

A considerable number of methods for the elimination of contaminants preparatory to cultivation have been devised. Their purpose is to kill the contaminating organisms and preserve the tubercle bacilli. They are based on the greater resistance of the tubercle bacillus; however, careful timing and experience are necessary to success because of variation in numbers of bacilli and in the character of different specimens of sputum. Corper's (1930) method employs 5 per cent oxalic acid; MacNabb (1936) recommends 3 per cent hydrochloric acid followed by 3 per cent NaOH to neutralize, using brom cresol purple as indicator. Sodium hydroxide 4 per cent and potassium alum 5 per cent are frequently used, followed by neutralization by hydrochloric acid with bromthymol blue or brom cresol purple as indicator. Many workers still use the old anti-formin method. "Tergitol 08" has been used by Petroff and Schain (1940) and is recommended by Gershenfeld (1945). Once treated, the material is heavily spread over the selected special media (Lowenstein, Petragagni, etc.) and incubated for several weeks. Guinea-pig inoculation is still essential in many cases to prove the presence of tubercle bacillus, and many laboratories get a much higher percentage of positives by animal inoculation than by culture.

#### EXUDATES FROM SEROUS CAVITIES

Smears made from the pus or from the sediment after centrifugation of fluids and stained by Gram's method should be studied to guide the selection of media and method. The clinical diagnosis must be taken into careful account because the film may not



show anything. Aerobic and anaerobic cultures on blood-agar plates and cultures in thioglycollate broth are as a rule sufficient for most of the common organisms causing acute types of serous effusions and exudates. If the infection of joint fluids with gonococcus is suspected, inoculation of special media (Peizer, 1942) and incubation in a 10 per cent CO<sub>2</sub> atmosphere must be added to the general procedure. "Meningococcus joints" are by no means uncommon but are seldom examined because the patient does not complain of them. The pus is of an apple-green color and must be examined as soon as the joint appears to be affected, because meningococci may be very numerous then and completely disappear in a few hours. The usual culture methods give good results. For the isolation of *Mycobacterium tuberculosis* from this type of specimen, and it is particularly indicated in pleural fluids without obvious cause, we strongly advise inoculation of guinea pigs in addition to, and, if necessary, in preference to, smears and cultures; smears alone are never satisfactory. If a clot forms it should be broken up and the material thus obtained used for the cultures and animal inoculation; it is seldom necessary to treat it to eliminate contaminants.

#### STOOLS

Specimens of stool for bacteriologic studies should be sent to the laboratory as quickly as possible after obtaining them in sterile containers with a well-fitting lid to prevent drying. In some instances, such as the dysenteries, overgrowth by coliforms prevents isolation of the pathogens. Specimens from lesions in "ulcerative colitis" are best collected by proctoscope and sigmoidoscope on suitable swabs, taken by an experienced clinician guided by the bacteriologist. Most stool specimens sent for culture come from patients with acute intestinal diseases; organisms of the *Salmonella-Shigella* group are then sought with media which

give a primary differentiation by presence or absence of lactose fermentation. In addition to the use of selective media, inoculation of blood-agar plates may prove helpful in cases of acute gastro-intestinal upsets (food poisoning) which may be caused by different bacteria or their toxins. If outbreaks of food poisoning occur it is very important to obtain immediately samples of the incriminated food as well as of stool and vomitus. Even the isolation of members of the *Salmonella* group may be difficult from the stool and more readily and more importantly done from the incriminated food; in either case, selective enrichment media (tetrathionate broth, S.S. medium) may be necessary. Staphylococci will grow from stools and foods on blood-agar plates, but the findings of large numbers of staphylococci is only suggestive presumptive evidence of their being the causative agent in a particular outbreak unless the strain is shown to produce enterotoxin. Unfortunately, no simple test for the presence of enterotoxin is available and it is necessary to resort to Dolman's (1936) or Hammon's (1941) tests on kittens.

Food poisoning due to the toxin of *Clostridium botulinum* presents a characteristic clinical picture; the laboratory confirmation depends on the isolation and identification of the organism by anaerobic methods or, more importantly, the demonstration of the type-specific toxin in the food and rarely in the filtrates of bowel contents.

In bacillary dysentery there is no difficulty in isolating the causative organism from a freshly passed characteristic stool within the first three days of onset. From the fourth day of disease onwards isolation becomes increasingly difficult and often is only possible on Leifson (1935) medium or its modification "S.S. medium" (1940), or on bismuth-sulfite medium. In any case, the whole stool (not a sample) should be sent to the laboratory as soon as it is passed and the pan must be free of disin-

fectant. If the laboratory is far distant the stool should be mixed with an equal volume of 0.2 per cent sodium hydrate to keep it alkaline and prevent overgrowth by *Escherichia coli*. Shiga, Schmitz, Flexner, Sonne, Madampensis and Newcastle types have to be differentiated; types can be recognized within the Flexner (*Shigella paradysenteriae*) species and can have epidemiologic significance. There is, too, some relation of the kind of organism to the severity of the disease; there can be mixed infections (including protozoal and others in the tropics) and in endemic regions there are other related organisms which cause confusion, particularly in late stages of the disease and towards the termination of an epidemic.

Although cholera presents a striking clinical picture, diagnosis may depend entirely on the laboratory. In cases of cholera diarrhea occurring in the course of an epidemic, in mild cases, in isolated cases arriving by ship, plane, etc., and in the initial cases of an epidemic which may have died before clinical diagnosis was possible, laboratory examinations are essential. The all-important procedure is the isolation and identification of the vibrio from stool, vomitus or soiled clothing or bedding. Microscopically, the typical vibrios are found abundantly in the mucopurulent flakes in characteristic stools, but they may be scarce in atypical cases. They grow very rapidly and readily at pH 8 in 1 per cent peptone water (even more dilute solutions may be used to discourage the growth of other organisms); the soft pellicle which forms at the surface is subcultured after 3 to 5 hours at 37° C. onto alkaline 3 per cent bile agar. Rapid transfers from the surface in alkaline peptone water may be necessary; bismuth-sulfite enrichment media are recommended by Read (1939) and Wilson and Reilly (1940). The peptone-water culture can be tested for the cholera red reaction and for other reactions. Blood cultures are useless, and titration of immune bodies in the patient's serum is of no particular help.

Pfeiffer's reaction (intraperitoneal bacteriolysis of vibrios by anticholera serum in the guinea pig) is often an essential means of identification. Water from suspect sources must be carefully skimmed from the surface and taken in shaded regions of open water systems, such as rivers, ponds, tanks and wells. Slime and mud exposed at low levels of water in the dry season and collected in well-shaded situations may prove important. Concentration by filtration of large volumes of water is also used. Dieu-donné selective medium (1909) must be allowed to "ripen" by taking up carbon dioxide and losing ammonia and should be used within 30 hours of pouring into plates.

Examinations of stool specimens for tubercle bacilli can be carried out by any of the methods described for sputum or stomach washings. In tuberculous ulceration of the lower bowel it is often possible to demonstrate the *Mycobacterium* in the mucus scraped from the surface of a formed stool. To do this the entire formed stool must be sent to the laboratory in the bedpan as passed, and the very thin usually invisible flakes of mucus must be removed from the surface by gentle scraping with a wire loop. The interpretation of microscopic finding of acid-fast bacilli in stools requires all the precautions mentioned under sputum examination, and proof of pathogenicity is desirable. The finding of tubercle bacilli in stools is not necessarily proof of intestinal tuberculosis, as any open lesion in the lungs or larynx can lead to positive findings in stools. In infants cultures of stools for tubercle bacilli instead of gastric washings have been suggested by some authors.

#### BILE

Anaerobic and aerobic cultures on blood-agar plates and cultures in thioglycollate medium and on media selective for the *Salmonella* group should be set up with all specimens of bile. The commonest purposes of cultures are to find the causative agent



of cholecystitis or to isolate *Salmonella typhosa* in suspected carriers. Bile is either obtained at operation or by drainage with a duodenal tube. This presents no great difficulty but it is necessary to separate the first intestinal running and to collect the bile specimen later.

#### SPECIMENS FROM THE UROGENITAL TRACT

Urinary tract infections contribute a large percentage of specimens sent to the laboratory, and their collection with essential precautions is sometimes troublesome because of reluctance to catheterize patients. Although reasonably useful specimens of urine can be collected by intermittent voiding and discarding the first running, catheter specimens are essential in many cases and always from females. The external genitalia must be carefully cleansed, and with particular care in female patients. The specimen must be inspected by transmitted light, its appearance noted and any mucopurulent flakes fished with a pipette or wire, because they often yield purer cultures of significant organisms than general cultures do. This is particularly true in chronic gonorrhea, when stained films too may be informative. Films made of the centrifuge sediment are stained by Gram's method; microscopic examinations for casts, red blood cells, pus and motility of organisms also give useful information and should not be neglected.

For the isolation of the common urinary pathogens the inoculation of thioglycollate broth, a blood-agar plate and one plate of a selective medium (MacConkey) is recommended, but for the isolation of *Neisseria gonorrhoeae* or *Brucella* appropriate media have to be used in addition. For the isolation of tubercle bacilli from the urine a 24-hour specimen should be collected, acidified slightly with acetic acid and allowed to sediment overnight after the addition of 3 cc. of 5 per cent tannic acid per liter. The super-

natant is decanted, and after concentration in the centrifuge the sediment is made alkaline to neutral red with 4 per cent NaOH and let stand for one-half hour at 37° C.; it is then neutralized with hydrochloric acid, and the sediment used for smears, cultures and guinea-pig inoculations. The ureteral catheter specimen should be cultured in thioglycollate broth and on a blood-agar plate overnight, even when examination for tubercle bacilli is requested; only if no growth occurs should it be injected into guinea pigs. Otherwise, if pathogenic organisms are present, the specimen has to be treated with one of the previously mentioned reagents prior to injection. Guinea-pig inoculation is recommended in favor of cultures as the specimens are usually very small and contain only very few mycobacteria.

Urethral secretion from the female, and sometimes from the male, may have to be collected on sterile cotton swabs; at least two swabs should be taken, one for smear and one for cultures. Aerobic cultures on blood-agar plates and on special media to be placed in 10 per cent CO<sub>2</sub> atmosphere and immersion of the swab in thioglycollate broth, will cover a fair range of pathogens. Prostatic secretion is specially collected in sterile tubes or Petri dishes. The meatus should be thoroughly cleansed and the urethra constricted by the patient during the prostatic massage so that as much material as possible is obtained at once. The collection of cervical discharges must be done through a speculum; after removal and discard of the mucus plug, the cervix is cleansed with sterile swabs; fresh sterile swabs are then gently rotated in the cervix and used for smears and cultures. When ever unusual infections are suspected, their examination should be specially arranged for with the laboratory to secure adequate specimens and immediate examination by suitable methods.

## MATERIAL FROM WOUNDS

This covers a wide variety of specimens and of infective organisms. The available amounts of exudate may be small and can then only be collected on sterile cotton swabs or with a platinum loop. If large quantities of pus or serous fluid can be obtained, a good representative amount should be collected, but it is well to remember that scrapings from the wall of an abscess or a sinus is more suitable and more reliable than old static pus from the lumen. At times, quantity is important, and in actinomycosis even the pus-saturated dressings should be sent to the laboratory in order that granules, which may be scarce or regional, may be washed out of them. The selection of a specimen from a particular spot is often most valuable in infected wounds, especially when secondary contamination prevails and may obscure the situation. The careful selection of specimens cannot be overemphasized, and consultation with the bacteriologist at the bedside is not only valuable in this respect but also promotes his interest in the case.

Any material from wounds should be put up on two sets of media for aerobic and anaerobic culture; examination of Gram-stained smears guides the selection of media and other necessary procedures. The laboratory must be informed of the source of material and most particularly of suspicion of gas gangrene, tetanus and actinomycosis, for the important organism may be missed if cultures are not put up in a suitable way and kept for a long enough time to produce recognizable colonies. Tuberculosis and diphtheria may be overlooked unless suspicion is directed towards these possibilities on clinical grounds. Material from uncontaminated tuberculous lesions may be cultured directly on special media or inoculated directly into guinea pigs, but it is very difficult to find acid-fast rods in smears of pus unless they are present in very large

numbers. The laboratory should be given fair warning if anthrax or any other highly contagious disease is suspected, as it is necessary to take proper precautions to protect the laboratory worker, to prevent disastrous infections in the animal colony and to preclude possible contamination of vaccines and other preparations.

## A SHORT OUTLINE OF PROCEDURES FOR THE IDENTIFICATION OF PATHOGENIC BACTERIA

## MORPHOLOGY AND COLONY FORM

In any attempt to identify one or several organisms grown from specimens, a regular procedure has to be followed to avoid missing the many possibilities, to provide essential information at the right time and to allow of final conclusions. The beginner will be particularly advised to adhere strictly to the definite scheme of procedure laid down in the laboratory and avoid devising short cuts. The experienced bacteriologist may occasionally be able to take a short cut and give at least a provisional opinion, but this should always be followed up with proof by subsequent tests.

The first important step is the study of the colony forms which vary in size, shape, color, texture and the degree of adherence to the medium, according to the kind of bacteria, the nature of the medium and the age of the culture. The maintenance of suitable standards in a laboratory allows the development of considerable skill and judgment on the part of experienced workers in the recognition of colonies and character of growth in fluid media, with great saving of time and of unnecessary reduplication of cultures. It is an important step towards the identification of bacteria by final criteria and is often assisted by alterations observable in particular media in the region of the colony, such as hemolysis, greening, soluble pigment, liquefaction, etc.



It may be helpful to students of bacteriology to list some of the commoner descriptive bacteriologic terms and the order of examinations in which these terms are used for the identification of unknown cultures with keys such as are found in textbooks and manuals of determinative bacteriology. For the most part, these terms are self-explanatory and only strange in their unfamiliarity, but others have acquired a strict technical meaning.

The appearance of the growth can be described in the following terms:

**Size.** Colonies are usually measured in millimetres, but are often loosely described as large, small, pin-point, etc.

**Form.** The shape may be round, irregular, vine-leaf, and in the depth of the medium may be lenticular, diffuse, filiform, beaded, papillate, villous, arborescent. The elevation may be effuse (flat), convex, umbonate, conical, raised, depressed; the margin may be entire, undulate, lobate, crenate, rhizoid, fimbriate, filamentous and spreading.

**Texture.** May be amorphous, granular, friable, brittle, dry, membranous, adherent, viscid, slimy mucoid, butyrous, dewy.

**Lustre.** May be dry, dull, moist, glistening, mirrorlike, iridescent.

**Opacity.** May be transparent, translucent, cracked-ice, opaque, or giving an iridescent change of color with variation in the incidence of the light.

**Color.** May vary greatly, from white through pale cream to yellow and orange and brown, greenish-yellow to bluish-green, pink and red to purple and black; there may be yellowish, green or bluish pigment soluble in the medium.

**Emulsification.** The growth may suspend in water easily and homogeneously or with difficulty and granularity or hardly at all in flakes.

**Degree of Growth.** None, scanty, moderate, abundant, profuse, spreading.

**Odor.** Absent, decided, putrid, resembling some known smell.

**Position.** Growth in fluid, semisolid or in shake cultures in agar may appear as uniform, surface, near the surface, at the bottom (deep); in fluid media, there may be a pellicle formed of characteristic density and structure, also the growth may be flocculent or granular and may sediment more or less completely as a viscid mass or as a flaky de-

posit; there may be a slime wall at the edge of the fluid surface or stalactite growth hanging from the surface film.

**Changes in the Medium.** Hemolysis, greening or no change in added blood, production of acid or alkali, liquefaction in a characteristic way, peptonization, coagulation, browning, reduction of dyes and colored by soluble pigments.

Colony form and growth characters should be noted down carefully after inspection and a hand lens should be used with both transmitted and reflected light.

The second step is the study of the morphology of the organisms which form the colony.

**Staining Reactions.** Gram positive, Gram negative or acid fast. With ordinary or special stains, the staining may be even, irregular, beaded, banded, septate; polar staining and granules may be present.

**Size.** Should be expressed in microns which can be measured or estimated by comparison with known material (red blood cells, etc.), but length and breadth are often expressed in very general terms.

**Shape.** Cocci, bacilli, curved rods, spirillum, spirochetes, filaments, branched or unbranched, ends round or pointed, or square, sides parallel or uneven or bulged.

**Arrangement.** Organisms may occur singly, in pairs, chains, cords, clusters, packets and special characteristic arrangements such as the "chinese letters," Vee form or palisade. The typical arrangement of the organism is better studied in fluid media than from a surface as there is less tendency to distortion.

**Structure.** The demonstration of well-formed capsules, for which it may be necessary to use special methods, such as Muir's (1916), Hiss' (1905) capsule stains, specific serum to cause swelling or animal passage. The formation of endospores which may be round or oval, large or small and may be terminal, subterminal or equatorial in situation. The fully formed spore may not distort the cell or it may bulge it so as to give it the appearance of a drumstick, club or a navicular shape. Presence of flagella can be demonstrated, and it is important to note their number and position (peritrichous or terminal) by the use of selective staining methods (Gray, 1926; Leifson, 1930). Motility observed in the living state and distinguished from Brownian movement, with other characters will indicate

whether flagella staining is necessary. It is also to be remembered that nonmotile variants of motile species do occur and that motility is often best observed in cultures grown at from 15° to 20° C.

### BIOCHEMICAL REACTIONS

These constitute the third step in identification of cultures and depend on the physiologic utilization of materials, on by-products and on secretions and excretions. Organisms which are morphologically indistinguishable are readily differentiated by these functions. Growth in the presence or absence of oxygen permits the division into strict aerobic and strict anaerobic organisms with intermediate facultative kinds, of varying degree, which constitute the majority of pathogenic bacteria. Thus, terms such as "facultative anaerobe," "obligatory aerobe," "micro-aerophilic," etc., indicate preferences and degrees of tolerance for the presence or absence of oxygen. Carbon dioxide requirements are strict in a very few instances.

At present, the most widely used criteria for identification are founded on the selective metabolic activities of bacteria. The chemical substances used by specific bacteria and the by-products demonstrable as the result of growth on media containing known substances have proved of great value. These constitute the fermentation tests, proteolytic tests, oxidation and reduction tests and tests of production of various substances. Most of them have the great virtue of simplicity of apparatus and reagents and of directness of application, so necessary to a very busy diagnostic laboratory receiving large numbers of specimens. Most are purely qualitative detection of changes, such as production of acid and gas from purified carbohydrates or alcohols or fatty acids, production of indol from tryptophane, liquefaction of gelatin, coagulation of milk and digestion of the clot, oxidation of potassium tellurite, reduction of nitrate to nitrite, and very many others of

equal simplicity and no less usefulness.

Yet other useful criteria depend upon testing the resistance and tolerance of bacteria to various physical and chemical agents. Resistance to heat is tested by placing cultures at carefully selected temperatures for a defined time, such as 10° C., 20° C., 40° C., for 24 hours, or 60° C. for 1 hour and 80° C. for one-half hour, and subsequently observing whether growth has occurred or whether the organisms have survived. Tolerance to strong salt solutions such as 6.5 per cent NaCl or to certain aniline dyes is used in distinguishing characters. Such tolerances can be used too for selective isolation of pure cultures from mixtures.

### IMMUNOLOGIC IDENTIFICATION

The recognition of antigenic structure has become the essential method for identification of species and for the determination of types within the species. In the *Salmonella* and *Shigella* groups this method is now indispensable, and for many other organisms it is a quick way of identification, provided that purified sera are available and proper controls are set up. Certain other established facts have to be considered to avoid grave errors. Cross reactions of certain types of pneumococci with types of *H. influenzae* (Alexander et al., 1946), of pneumococcus type 2 with Friedländer type B (Avery et al., 1925), the occurrence of a common antigen in various *Salmonella* species, all introduce complications which have to be taken properly into account. Unless bacterial morphology and staining reactions, cultural requirements and physiologic criteria are used as guiding principles, serologic reactions may lead to confusions. The value of serologic typing as a quick diagnostic procedure is discussed in the special chapters dealing with particular organisms and the diseases they cause. Identical techniques are applied in different instances, and examples of these are: capsule swelling for identifying types of pneumococci, of *H. in-*



*fluenzae*, of Friedländer's bacilli and sometimes of meningococci and other bacteria; agglutination for the recognition of species and for typing of *Shigella*, *Salmonella*, *Meningococci* and others; antitoxin protection tests in diphtheria, botulism and others; precipitin tests for the Lancefield (1933) grouping of *Streptococci* using the specific polysaccharide extracted by Fuller's (1938) or by Brown's method (1938), typing of *Streptococci* according to Griffith (1935). Unfortunately, all laboratories are not able to apply every one of these serologic tests as the highly purified specific monotype sera are not readily available. Many of them cannot be bought, and few laboratories have the personnel, time and equipment for their production. Central supply stations, on a national basis, which could provide reliable sera are urgently needed.

Even serologic typing has limitations as is illustrated by the typing of typhoid cultures, which can be separated only on the basis of their selective susceptibility to type specific strains of bacteriophage (Craigie, 1938).

Besides providing means of quick exact identification, the typing of organisms is of tremendous value in epidemiologic studies and in specific serum therapy. Its application to the study and management of disease continues to be of paramount importance.

#### PATHOGENICITY TESTS

Inoculation of suitable animals has several applications each of which has a proper place in the scheme. It is necessary for determination of the virulence of cultures capable of causing experimental infections; it is a means of identification on the basis of characteristic lesions and of protection tests using known antisera. Conversely it serves to recognize or titrate antibodies in the patient's serum by protection tests, and finally it is a method of isolation of organisms from infected material when culture methods are difficult or impossible. Inoculation is per-

formed either subcutaneously, intramuscularly, intravenously, intraperitoneally or intracerebrally in guinea pigs, rabbits, white mice or white rats according to the test in question and the susceptibility of the animal.

Various illustrative examples may be given. The only certain method by which the identity and virulence of *Corynebacterium diphtheriae* can be confirmed is by inoculation of guinea pigs. A suitable method is the subcutaneous injection of a saline suspension of a pure culture from an 18-hour Loeffler slant into each of 2 guinea pigs, the second of which (control) received 250 to 500 units of antitoxin on the previous day. The unprotected animal will die within 2 to 5 days if the growth is of virulent *C. diphtheriae*, with large hemorrhagic adrenals and a gelatinous oedema at the point of injection. Antitoxin-protection tests in animals (mice and guinea pigs) determine the types of botulinum toxin and of *Clostridium perfringens* (*welchii*) toxin and identify other toxins. An intraperitoneal bacteriolytic test using known serum identifies *Vibrio cholerae*. Characteristic lesions are important for the identification and for the isolation of *Leptospira icterohaemorrhagiae*, *Bacillus anthracis* and can be of great assistance with *Brucella* and *Pasteurella*. To establish the identity and virulence, or isolate tubercle bacilli in doubtful cases, guinea pigs are injected intramuscularly with a saline suspension of the growth on media, or of suspect material from patients. In positive cases the guinea pig will die within 4 to 8 weeks with typical lesions in which characteristic acid-fast rods can be seen and cultured. The difference in the lesion produced by a standard dose of culture in the rabbit distinguishes the human type from the bovine type of *Mycobacterium tuberculosis*. Animal inoculation is the only means of isolating and identifying *Spirillum minus* of rat-bite fever. Tests for the titration of fractions of units of antitoxins in the serum of patients are done by

intradermal inoculation of animals with suitable mixtures.

It is not to be lost sight of that the laboratory animal, especially the rabbit, is the source of many antisera required for the immunologic identification and typing of bacteria. Also, the study of disease processes is greatly facilitated when a susceptible experimental animal is available. The question of susceptibility limits the application of the method which is far from being universal. It is well to emphasize, too, that special precautions are necessary to protect the workers and the animal colony when animal inoculations and post-mortems are done with highly infectious material.

## A SIMPLIFIED GUIDE TO THE PROVISIONAL RECOGNITION OF COMMON GROUPS OF BACTERIA

The purpose of the following listing of selected characters is to enable inexperienced workers to recognize the main groups of commonly found organisms. Once such a group has been determined reference can be made to the appropriate chapter of this book and to tables and descriptions in books such as Bergey's *Manual of Determinative Bacteriology* for final identification. This list is not a determinative key but is the utilization of common simple characters as a first step in practical procedure for any scheme of bacteriologic diagnosis. Only commonly found bacteria have been included; less common and unusual forms have been left out for the sake of simplicity.

### I. Aerobic cultures.

#### 1. Gram-positive cocci.

- A. Arranged in clusters, form large pigmented opaque colonies: *Staphylococcus*. Distinguish pathogenic from nonpathogenic species by plasma coagulase and biochemical characters.
- B. Short and long chains and even pairs in pus—*Streptococcus*.

- a. *Pyogenes* Group: distinguish by hemolysis, Lancefield grouping. Does not grow at 45° C.
- b. *Viridans* Group: greenish of blood, not soluble in bile, do not ferment inulin; grow at 45° C. and not at 10° C.
- c. *Enterococcus* Group: some hemolytic and some no action on blood; grow at 10° C. and 45° C. and survive 60° C. for 30 minutes. Grow in 1 per cent methylene blue and in 6.5 per cent NaCl. All are Lancefield Group D.
- d. *Pneumococcus*: greenish of blood (Hemolysis in Anaerobic culture). Soluble in bile, well-marked capsule, ferment inulin. Typing by capsule swelling with specific antiserum.

#### 2. Gram-negative cocci, mostly in pairs.

- A. *Neisseria intracellularis*: requires enriched media, ferments dextrose and maltose with acid production; saccharose, lactose and levulose not fermented. Agglutination by type antimeningococcus serum, and if not available by polyvalent serum.
- B. *Neisseria flavescens*: requires enriched media, yellow colony, no carbohydrates fermented, no agglutination with antimeningococcus serum.
- C. *Neisseria gonorrhoeae*: requires specially enriched media, ferments dextrose only. Positive oxidase test helps isolation from mixed cultures from cervix. The alkali-solubility test described by Cantor et al. (1942) is important. No agglutination with meningococcus sera.
- D. *Neisseria catarrhalis*: grows well on ordinary culture media, no fermentation of carbohydrates.
- E. *Pharyngeal group*: (*N. sicca*, *N. flava*, *N. perflava*, *N. subflava*) grow well on ordinary



culture media, pigment production is best on Loeffler's slant, distinguished by biochemical reactions.

### 3. Gram-negative rods.

A. Grow freely on ordinary media, ferment carbohydrates, no capsule. Motile and nonmotile (peritrichous flagella).

a. Lactose positive—*Coli-aerogenes* group.

b. Lactose negative—*Salmonella-Shigella* groups.

Differentiate members of the groups by biochemical tests and specific antisera.

B. Some requirement of special media and conditions, no fermentation of carbohydrates, no capsule, nonmotile—*Brucella*. Members of this group have to be differentiated by CO<sub>2</sub> requirement, H<sub>2</sub>S production, dye inhibition test and agglutinin absorption tests.

C. No growth or poor growth without special growth factors in the media. Capsule variable, nonmotile—*Hemophilus* and *Moraxella*.

a. *H. influenzae* shows characteristic satellitism along staphylococcus streak or other colonies, no growth on plain agar, encapsulated strains (mainly from C.S.F.) identified by type specific antisera.

b. *H. pertussis* shows characteristic colonies on Bordet-Gengou medium, agglutination by specific antiserum.

c. *H. ducreyi* cultivation rarely done for diagnostic purposes (clotted rabbit, sheep or human blood heated at 55° C. for 15 minutes is inoculated with scrapings from suspected lesions or pus from lymph nodes and incubated at 37° C. for 24 hours. Gram-stained films of the serum show small Gram-negative bacilli in chains).

d. *H. para-influenzae* grows on plain agar along a staphylococcus streak or other colonies providing "V" factor.

e. *Moraxella lacunata* from angular conjunctivitis, no growth on blood agar, characteristic pitting on Loeffler's serum slant; typical diplobacillus.

f. *Moraxella liquefaciens* from corneal ulcers, grows on blood agar, extensive liquefaction of Loeffler's serum slant; typical diplobacillus.

Other members of this group are not frequently encountered and have to be differentiated by special tests.

D. Large capsule, free slimy growth, fermentation of carbohydrates—*Klebsiella pneumoniae*. Capsule swelling with specific antisera, A, B and C (Julianelle, 1926); other strains have to be identified by biochemical tests.

E. Polar staining, grows on ordinary media but may need blood, and one member requires special media (*Past. tularensis*), ferments carbohydrates, no coagulation of milk—*Pasteurella*.

F. Polar staining, grows readily on ordinary media, honeylike colonies on potato, no fermentation of carbohydrates, slow coagulation of milk—*Malleomyces*.

G. Characteristic spreading growth, ferments carbohydrates, splits urea, motile with peritrichous flagella—*Proteus*.

H. No fermentation of carbohydrates, motile with polar flagella, many have soluble pigment—*Pseudomonas*.

No pigment, milk alkaline—*Alkaligenes fecalis*.

I. Pigmented colonies, otherwise like colon group—*Serratia*.

J. Grows readily on simple alkaline media, curved rods, actively motile with polar flagella

—*Vibrio*. Identification of *Vibrio cholerae* by agglutination with O group I antiserum (Gardner and Verkstranen, 1935; Linton, 1940) and biochemical tests, saccharose and mannite fermented, arabinose not fermented, no hemolysis of goat red blood cells—Pfeiffer's test with specific serum.

#### 4. Gram-positive rods.

- A. Nonmotile, arranged in Chinese figures, stain unevenly with bands and granules—*Corynebacterium*.

Three species of medical importance: *C. diphtheriae*—specific toxin. *C. pyogenes* and *C. ulcerogenes* differentiated by biochemical reactions and animal inoculation.

- B. Nonmotile, beaded staining, acid fast—*Mycobacterium*.

- a. *Mycobacterium tuberculosis* hominis and bovis are the 2 types of importance. Distinguish from saprophytic members by cultural characters and pathogenicity tests.

- b. *Mycobacterium leprae* cannot be grown in culture, not pathogenic for laboratory animals, acid-fast stains of smears of nasal secretions or skin lesions show vast numbers cramming the so-called lepra cells.

- C. Spores formed, usually without distortion of cell—*Bacillus*. One species of medical importance, *Bacillus anthracis*, grows well on simple culture media, characteristic colonies on agar, nonmotile, causes fatal septicemia in laboratory animals, capsule formed in animal body.

## II. Anaerobic cultures.

### 1. Gram-positive cocci.

- A. Occurring mainly in clusters but also in pairs—anaerobic *Staphylococcus*.

- B. Mainly in pairs and chains—anaerobic *Streptococcus*. Identification by growth characters and biochemical reactions—see

special chapters and Bergey's *Manual*.

### 2. Gram-negative cocci.

- A. Occurring in pairs with adjacent sides flattened—anaerobic *Neisseria*.

- B. In irregular masses, very small cocci—*Veillonella*, both types often found in tooth abscesses and in infections of genitourinary tract. For identification and differentiation—see Bergey's *Manual*.

### 3. Gram-negative rods.

- A. Motile or nonmotile, of varying sizes and shapes, non-spore-forming, often foul smelling—*Bacteroides*.

- B. Large pointed ends, effuse colonies, and difficult to grow—*Fusiformis*.

4. Gram-staining variable, true mycelium produced, true branching, non-acid-fast—*Actinomyces*. Identified by cultural characters. (Slow-growing colonies on solid media dry, crumbly resembling tubercle colonies, in fluid medium granules adherent to walls of tube. Unstained preparation of crushed granule shows typical clubs.)

5. Gram-positive rods: motile and nonmotile forming endospores which distort the cell, some species microaerophilic—*Clostridia*. Many members of this group are saprophytes but some are highly pathogenic for man and animals. Differentiation by biochemical reactions and pathogenicity tests and specific toxins.

## SPECIAL AND INDIRECT METHODS OF DIAGNOSIS

Isolation of the causative organism may fail when healing has taken place, or when the organisms have been suppressed by treatment before the specimens were taken, or when culture methods are not effective enough to permit growth from very small numbers of bacteria. Under these conditions, it becomes important to recognize the antibodies formed during the course of infection, or the antigens still present in body



fluids or excretions, as a means of indirect diagnosis.

#### ANTIBODIES IN THE PATIENT'S SERUM

**Agglutination.** Titration of the patient's antibody by agglutination of known cultures has proved of value in certain diseases. The classic example is the Widal test, introduced originally for the diagnosis of typhoid fever after the second week of disease. This method is based upon the determination of the greatest dilution of the patient's serum which will cause agglutination of a known bacterial antigen prepared in a selected standard way. There are several technics using macroscopic methods in test tubes or capillary tubes, or on slides and ruled plates of glass, and microscopic methods. Usually macroscopic methods carried out with reasonable measurable volumes in test tubes are most satisfactory; the other methods are desirable only when very small quantities of material are available. Agglutination titers vary considerably according to the technic used and comparisons can be made only between tests carried out by the same technic. Titers also differ between tests because of unexplained variation in agglutinability of different cultures of the same strain. This can only be properly overcome by assigning an "agglutinability factor" to each batch of suspension, as devised by Dreyer (Gardner, 1920), or by the more exact and elaborate determination of agglutinin nitrogen which is too laborious for a routine diagnostic method. Methods using living cultures are too variable from test to test to be reliable.

Serial dilutions of the patient's serum are made in a row of tubes and in a standard volume (1 cc.) to which a standard volume (1 cc. or 1.5 cc.) of standardized formolized suspension of killed known culture is added; this mixture is incubated at 55° C. and in some tests 37° C. in a waterbath for 6 hours or in some tests for 24 hours. The greatest dilution showing agglutination to the se-

lected end point gives the titer. For more accurate comparisons this may be reduced to the standard agglutination titer by the use of the agglutinable factor.

The specificity of the reaction is relied on for the diagnostic value. But as some "natural agglutinins" are often encountered, a threshold titer is usually accepted which must be exceeded for the test to be of diagnostic significance. Furthermore, antigenic analysis and studies of bacterial variation have established importance of "H" (flagellar) and "O" (somatic) agglutination in the recognition of antigens and antibodies, and this knowledge has to be taken into account in diagnostic tests. There is now general acceptance that "O" agglutination is of most significance in the serodiagnosis of active typhoid fever and other *Salmonella* infections, whereas "H" agglutination is more indicative of immunization by vaccines or of past illness. However, in certain specific infections, because of cross immunity with common antigen, special knowledge of the antigenic constitution of the organisms involved is required to interpret the results of "H" and "O" agglutination. It is always advisable to construct a curve of agglutination titers, determined by 3 tests performed at intervals of a few days, when a rising curve of specific antibody titer allows of more certain interpretation.

The agglutination test is useful in the diagnosis of typhoid fever, salmonella infections, brucellosis, tularemia, Weil's disease, and typhus. It is of very doubtful value in shigella dysenteries. Agglutination of "H" and "O" antigens of typhoid and paratyphoid in serum dilutions over 1:80 is considered to have diagnostic significance, unless vaccination has taken place recently. Agglutination of the "H" antigen only is rarely found in active cases but frequently in carriers, after vaccination, or in individuals who have had typhoid fever in the past. The duration of illness is an important factor, and it may be necessary to repeat the test in order to observe a rise or fall in

titer before any final diagnostic conclusions can be drawn.

In brucellosis, the agglutination test is of even greater importance as aid in diagnosis because the isolation of *Brucella abortus* is often unsuccessful. The bacterial suspension must be made from a smooth strain and tested for its agglutinability by known positive and negative sera; it must be heat and salt stable. A wide range of serum dilutions should be made because "prozone" reactions are quite common; incubation for 48 hours is necessary. It is very important that blood for the test be drawn before skin tests are performed or treatment with vaccine begun, as either of these procedures may cause a false positive reaction. In the interpretation of the results of *Brucella* agglutination tests, occupational groups which have a high rate of exposure, butchers, meat packers, dairymen, farmers, veterinarians and drinkers of raw milk, have to be taken into account. In these a positive agglutination of *Brucella* in a relatively low serum dilution of 1:80 or 1:100 may indicate only some past or subclinical infection (Evans et al., 1938; Huddleson, 1939); while the same titer in other population groups is suggestive though not indicative of active infection. Usually the results of other tests have to be taken into account (see interpretation of tests by Huddleson, 1939), as a negative agglutination test does not exclude active infection with *Brucella*. As further aid in diagnosis of brucellosis the opsonocytaphagic test is helpful, but requires considerable experience to give reliable results.

If the isolation of *Pasteurella tularensis* is unsuccessful, or cannot be attempted for lack of facilities, the agglutination test can aid in the diagnosis of tularemia if antigen is available. Francis and Evans (1926) concluded that the agglutination test in tularemia is very reliable and that only cross agglutination with *Brucella abortus* and *melitensis* have to be excluded. Sera should be set up against all 3 antigens so that the

difference in titer may permit a correct conclusion. If high titers against all 3 organisms are obtained, agglutinin absorption tests are necessary for differentiation.

The causative agents of Weil's disease, *Leptospira ictero-haemorrhagiae* or *Leptospira canicola* can be found in the blood during the first four days of disease and in the urine for 8 days (by guinea-pig inoculation). Agglutinating and lytic antibodies appear after about a week and increase and may persist for many years. Antigens consisting of formolized suspensions of the two strains of *Leptospira* and patient's serum, in varying dilutions, are mixed on slides and observed for agglutination either macroscopically or under the microscope. If cultures of living organisms are available the lytic power of the serum can be demonstrated under the darkfield microscope. The macroscopic technic of Smith and Tulloch (1937) gives best results and can easily be provided if there is sufficient demand for it. The persistence of antibodies long after the clinical signs of illness have disappeared makes it necessary to consider the whole course of the disease along with the positive agglutination or lysin test, in order to avoid erroneous conclusions.

The Weil Felix (1916) test for typhus may be mentioned here because a bacterium (*Proteus*) having a common antigen with the *Rickettsia* is used as antigen in the diagnostic test. The antigen must be prepared from a reliable strain of *Proteus* OX19, OXK, etc., and the technic of the test is the same as for bacterial agglutination tests.

Agglutination of antigens other than bacterial are used as confirmatory or diagnostic tests. One example is the agglutination of sheep red blood cells by the serum of patients with infectious mononucleosis (Paul and Bunnell, 1932). Sera from healthy people or those suffering from serum sickness quite frequently agglutinate sheep red cells but usually in low titer (1:40 or 1:60). It is important to distinguish these agglutinins from the antibodies which develop



in the course of infectious mononucleosis. Bailey and Raffel (1935) found that this could be done by absorption tests. The antibody of normal serum is absorbed by guinea-pig kidney. Other tests based on the agglutination of human red blood cells and those of other species are also used as diagnostic aids.

**Complement-fixation** tests are very useful as diagnostic aid in many diseases. The classic example is the Wassermann test, and its modifications, for the diagnosis of syphilis. It is beyond our scope to go into details of technic, and the reader is referred to special books on this subject. Complement-fixation tests are used extensively for the diagnosis of virus and rickettsial diseases. In some diseases of bacterial and protozoal origin, complement-fixation tests are used as a diagnostic aid, but the results can be evaluated only in conjunction with clinical and other bacteriologic studies. In acute uncomplicated gonococcal infection of short duration the test is almost always negative and so does not exclude gonorrhea. In long standing infections, particularly accompanied by complications (epididymitis, salpingitis, arthritis, etc.), a positive reaction occurs and is of diagnostic significance. However, a positive test is not necessarily indicative of prevailing disease, as it may remain positive for months or even years after complete cure.

Complement-fixation tests are occasionally used with limited success in whooping cough, glanders, trichinosis, *Echinococcus* infection and malaria (Mayer and Heidelberger, 1946).

**Precipitation tests** are only occasionally used as a diagnostic method, except in syphilis. In this disease the various types of precipitation or flocculation tests are more widely used than even complement fixation because of their relative simplicity. Precipitin reactions for the detection of meningococcus antigen in the cerebrospinal fluid are useful in cases when the meningococcus cannot be grown easily. The cerebro-

spinal fluid is put up in serial dilutions and a suitable dilution of potent antimeningococcus serum added; precipitation will be observed in an optimal zone. Antigens (such as pneumococcus polysaccharide) can be demonstrated in the urine by precipitin reaction.

Precipitin reactions have important applications in forensic medicine and in public health for the identification of species origin of blood and tissues and of adulteration of food products. They also allow the identification of blood-sucking insect meals.

### SKIN TESTS

The intracutaneous injection of suitable specific antigens is widely used in diagnosis; it depends on the local inflammatory response to the reaction between antigen and antibody. With toxic antigens, which of themselves induce injury and inflammation, the presence of antibody is determined by their neutralization and the consequent lack of erythema or other manifestation. In the latter instance some degree of quantitative estimate can be made by the amount of toxin which fails to cause reaction on injection. In every case experience has dictated the suitable dose to be injected, the size and character of reactions which are significant and its time of appearance and regression. It will suffice to enumerate here the most commonly used skin tests.

The **Schick test** purposes to indicate blood levels of diphtheria antitoxin (1/250 Unit per cc. of serum is sufficient to protect against the disease).

The **Dick test** is used to detect *Streptococcus pyogenes* antitoxin as an index of immunity to the erythrogenic factor in scarlet fever.

The **Schulz-Charlton reaction**, the converse of the Dick test, is used for the diagnosis of scarlet fever and depends on the local blanching of the rash by the injection of antitoxin.

The **Brucellergin test**, carried out with the nucleoprotein fraction of brucella (Huddleson, 1939) or the injection of brucella vaccine, is employed for the diagnosis of brucellosis. The reaction is read after 48 to 72 hours. Erythema with induration of at least 1 cm. in diameter represents a positive test; evaluation should be done in combination with results of other tests for brucellosis.

**Tuberculin Test.** Koch's Old Tuberculin, bacillary emulsion and the newer purified protein derivative (P.P.D.) are derived from cultures of *Mycobacterium tuberculosis* and are used as a diagnostic aid in suspected cases of tuberculosis. They indicate an allergy to the protein fraction of the organism but do not distinguish between past and present infection (Chapter 12).

The **skin test for tularemia** uses bacterial vaccine. Foshay (1940) claims positive results in 92 out of 100 cases in the first week of disease.

The **Ito (1913)—Reenstierna (1924) reaction** for the diagnosis of chancroid uses *H. ducreyi* vaccine. The test remains positive for years after recovery, a fact which has to be considered in its evaluation.

Diagnostic skin tests are also used in several fungus diseases. Skin tests require considerable care in the performance of an apparently simple technic, as well as controls and judgment in their interpretation.

#### DARKFIELD MICROSCOPY

This method is particularly helpful in the diagnosis of spirochetal diseases. It is carried out either directly on material from the patient or indirectly by examining material from inoculated animals. Darkfield is the method of choice in the diagnosis of syphilis, particularly in the early stages before serologic tests are of any value. The suspected lesion should be cleaned with physiologic saline after removing any crusts or pus which may be present due to secondary infection. The base of the lesions may be com-

pressed until a few drops of clear fluid can be collected; or the chancre can be induced to ooze freely by swabbing it with a little alcohol and waiting a few seconds. A drop of fluid is collected on a clean coverslip which is put firmly (observe Newton's rings) on a clean glass slide; it is then observed under the darkfield microscope. If no darkfield is available the clear fluid can be drawn into a fine capillary pipette sealed with paraffin or in the flame and sent as quickly as possible to the laboratory. Several slides should be prepared in the described manner, as occasionally the *Treponema* are few in numbers and might be missed in a single preparation. If any antiseptics have been used locally it is preferable to examine aspirations from regional lymph nodes. The skin is sterilized, the gland immobilized with the fingers and punctured with a 20- to 28-gauge needle attached to a 2 cc. syringe which contains about  $\frac{1}{2}$  cc. of sterile physiologic saline. The needle is gently rotated inside the lymph node and some of the saline injected; some fluid is then aspirated and examined in the same way as chancre fluid. The lymph node puncture is preferable to direct examination of lesions in the mouth because certain oral spirochetes are morphologically indistinguishable from *Treponema pallidum*. A substitute method is to add the fluid to a small drop of India ink (Burri's method), or to 1 per cent nigrosin, or to 2 per cent Congo red blued with acid after completing the preparation. The mixed drop is rapidly spread as a thin film and quickly dried in air. It often shows the spirochetes perfectly.

Darkfield examination of blood reveals the *Borrelia* during a febrile attack of relapsing fever; when few in number their violent motion aids in finding them. White mice can be injected with the blood and will show large numbers of *Borrelia* when their blood is examined later in the darkfield.

Rat-bite fever is thought to be caused by at least 2 organisms, *Streptobacillus moniliformis* and *Spirillum minus*. The former



can be cultivated from the blood, the primary lesion or enlarged lymph nodes. To detect *Spirillum minus* blood or exudate from the primary lesion or material aspirated from the regional lymph node should be injected into guinea pigs. The blood of the guinea pig should be examined daily under darkfield from the fifth day after inoculation. Few other organisms are as rapidly motile; they can also be stained with gentian violet.

*Leptospira icterohaemorrhagiae* is practically never found in the blood or urine of patients by direct examination, but if blood or urine containing them are injected into guinea pigs, the leptospira can be demonstrated by darkground illumination in the animals' blood or in suspensions of liver or kidneys after the animals' death. Fresh fluids should be examined whenever possible to see the characteristic movement of the organism.

Coles' method (1915) is a very elegant and helpful method of finding spirochetes, especially when they are scanty in the fluid to be examined. Thick films are made and dried in air as quickly as possible, blood is dehemoglobinized with distilled water after drying, and fixed with alcohol. The preparation is stained with any Romanowsky stain (Leishman, Wright, Giemsa, etc.) and examined under darkfield illumination. The spirochetes often do not stain well enough to be seen by direct transmitted light, but they are brilliantly and beautifully opalescent in the darkfield with the colors of a fire-opal, and very easily seen.

#### ANIMAL INOCULATION AS AN AID TO DIAGNOSIS

This method has been discussed in previous sections and is mentioned here once more because in many instances it is the only one by which a diagnosis can be established or proof obtained of the pathogenicity of isolated organisms. To recapitulate the most common important uses: iso-

lation of *tubercle bacilli* in guinea pigs, of *Pasteurella tularensis* in guinea pigs or white mice, of *Malleomyces* in guinea pigs, of *Bacillus anthracis* in guinea pigs or white mice, of *Leptospira icterohaemorrhagiae* and of *Spirillum minus* in guinea pigs, of *Brucella abortus* in guinea pigs; the identification of the toxins of *Corynebacterium diphtheriae*, of *Clostridium botulinum* and of *Clostridium tetani* in the guinea pig, and the demonstration of *Staphylococcus enterotoxin* in kittens. It is thus clear that a well-kept and well-stocked animal house is a necessity for a diagnostic laboratory.

#### PRECAUTIONS AND TESTS IN RELATION TO ANTIBIOTICS

The general use of sulfonamides and of penicillin and streptomycin in the treatment of infections makes certain precautions and tests a necessity in the clinical laboratory. Only the everyday practical applications will be discussed here under three headings.

**The Neutralization of Sulfonamides and Antibiotics in the Various Specimens Sent for Culture.** This is a very important step, and the information as to the type and amount of drug received by the patient before the specimen was taken must be furnished by the physician. As pointed out earlier, the growth-inhibiting action of the sulfonamides can be neutralized by para-aminobenzoic acid, that of penicillin by penicillinase or clarase and that of streptomycin by cysteine (Denkelwater et al., 1945). As only the still viable organisms grow after successful neutralization, it is quite common to find organisms on smears while the cultures remain sterile.

**The Testing of Isolated Organisms for Their Susceptibility to Antibiotics.** This test should be carried out as rapidly as possible after the isolation of the bacteria because on it depends the choice of the therapeutic agent and to some extent the amount to be given. Rough qualitative tests can be

carried out easily and quickly with paper disks soaked with known amounts of the agent and stored in the ice chest (Morley, 1945). The disks can be impregnated with varying amounts of the antibiotic to give an indication of the relative sensitivity of the organism. To save time the disks can be placed on the plate right after inoculation if pure cultures or only slightly mixed primary cultures are expected, but in heavily mixed cultures it is essential to isolate each pathogenic organism separately and test it individually. This rough estimation of sensitivity can be followed by more exact quantitative tests whenever indicated.

**Estimations of Antibiotic Levels in Various Body Fluids.** Most of the fluids have to be diluted within certain ranges which depend on the amount of antibiotic received by the patient, and in the case of

intermittent injections, on the time between the last injection and the taking of the specimen. The mode of treatment (local, intravenous, intramuscular, intrathecal, etc.) has also a definite effect. For instance, it is important for the laboratory to know if an antibiotic was given intravenously or intrathecally if its content in spinal fluid is to be estimated. All this information should be clearly and precisely furnished by the clinician to the laboratory, which can then make appropriate arrangements. All fluids to be studied for drug concentration should be taken under aseptic conditions into sterile containers because all the biologic methods are based on the inhibitory action of the fluids on a standard test organism and their results are therefore confused by contamination.

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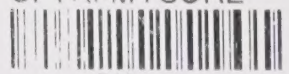
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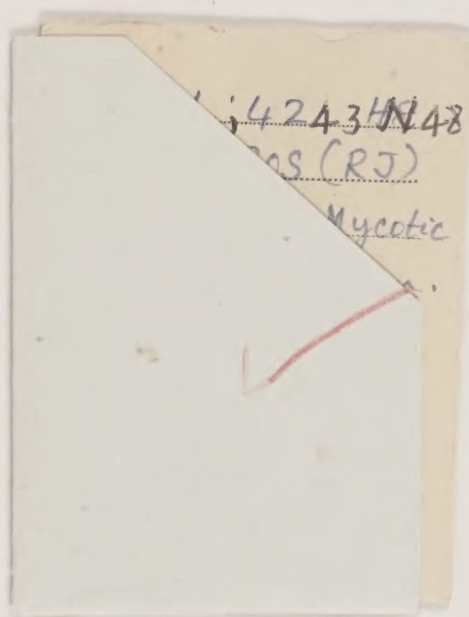
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